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The sea turtles of São Tomé and Príncipe: Ecology, genetics and current status of distinct species nesting on an oceanic archipelago

"Documento Definitivo"

Doutoramento em Biodiversidade, Genética e Evolução

Joana M. Hancock

Tese orientada por:
Professor Doutor Rui Rebelo e Professor Doutor Nuno Ferrand

Documento especialmente elaborado para a obtenção do grau de doutor

2019

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Documento especialmente elaborado para a obtenção do grau de doutor

Trabalho apoiado pela Fundação para a Ciência e Tecnologia (FCT) através da atribuição de uma bolsa de doutoramento (PD/BD/52599/2014)

2019

This dissertation should be cited as:

Hancock JM (2019) The sea turtles of São Tomé and Príncipe: Ecology, genetics and current status of distinct species nesting on an oceanic archipelago. PhD Thesis, Universidade de Lisboa, Portugal

Nota Prévia

A presente tese apresenta resultados de trabalhos já publicados ou em preparação para publicação (capítulos 2 a 4), de acordo com o previsto no nº 2 do artigo 25º do regulamento de Estudos Pós-graduados da Universidade de Lisboa, publicado no Diário de República II série 2.ª série — N.º 60 — 26 de março de 2018. Tendo os trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, Janeiro de 2020

Joana M. Hancock

To Chencho Castillo and all the old turtle hunters from land and sea who always saw the beauty of turtles like no one else and taught me in gentle and inspiring ways to love these amazing creatures even more.

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ACKNOWLEDGEMENTS

First, I acknowledge a fact: this work was only possible due to the financial support of FCT through an individual doctoral scholarship (PD/BD/52599/2014), and numerous sponsors of both sea turtle conservation programs in São Tomé and Príncipe.

None of the work presented would have been possible without the extraordinary work of the team members of Programa Tatô, in São Tomé, and Programa Têtuaga in Príncipe. These teams, led by outstanding and inspiring women such as Sara Vieira and Vanessa Schmitt, are composed of many men and women who work 365 days a year for marine turtle conservation in an archipelago that is blessed by outstanding natural beauty, but plagued with poverty and difficult living conditions. Most data and samples were collected by program's staff. I am forever in debt with them.

All the in-water work was conducted by teams of turtle fishermen, who were active when I first contact them. I was so proud and humbled to see how their initial disdain and suspicions of me and my work slowly but steadily gave way to a beautiful friendship based in trust and respect and a shared dedication to sea turtle conservation. Obrigada Lúcio Taraveira, à sua família, e ao Didy por toda a amizade e dedicação. Logistic support for in-water work conducted in the south of São Tomé was provided by the NGO Marapa.

Yaiza Yanes Perez not only endured extremely difficult living conditions, food of doubtful quality, endless diseases and many sleepless nights to assist me collecting most of the green turtle samples used in this study, but also gave me endless evidences of an enduring friendship that started more than 15 years ago, and a true passion for sea turtle conservation. GRACIAS GUAPA!

I would like to express my immense gratitude to my official and unofficial supervisors Rui Rebelo, Nuno Ferrand, Helena Gonçalves and Fernando Sequeira, and finally last but not least, Marc Girondot. The first for being the best supervisor a student could ask for (even all the times he made me cry and being down to earth and cold when I presented by crazy ideas), the second for accepting me with arms wide open into his lab, and providing me a lab and a team that one could only dream of. Helena and Sequeira, who had much more to do than to put up

with studies about turtle genetics, were very brave to accept me as their student to guide me through the meanders of genetic analyses.

The genetics lab work was mostly carried out by the staff of Centro de Testagem Molecular (CTM) in Vairão; Susana Lopes and Patrícia Ribeiro were not only excellent teachers and mentors, but real “turtle mothers” but prevented nervous breakdown genotyping the several hundred “hijos” and genotyping numerous sequences.

My mental sanity and motivation were largely ensured by the best family and friends one could have, including my mother, my sisters Sofia and Mariana; Tiago, my best friend these long 4 years, and of course, my dog Orelhas, natural of Príncipe island.

Finally, I am where I am today because the two most important men in my life, who never ever doubted of me and always fought to give me all they could to ensure I would get an education and pursue my dreams. My late grandfather Harry and Dad. Ultimately, this work is for them, and because of them.

RESUMO

O declínio populacional das tartarugas marinhas em todo o mundo, impulsionado pela caça excessiva, a perda de habitat e outros factores antropogénicos tornaram estes animais uma prioridade global de conservação. As tartarugas marinhas são particularmente susceptíveis a perturbações antropogénicas e naturais devido ao seu ciclo de vida longo, definido por comportamentos complexos tais como a migração entre zonas de alimentação e reprodução e filopatria de adultos, migrações de juvenis e uma forte influência de factores abióticos na disponibilidade de recursos, sucesso reprodutivo, e proporção entre os sexos ao nascimento.

O estado das populações de tartarugas marinhas é avaliado de acordo com o índice de abundância populacional, que no caso destes animais, se traduz tipicamente no número de ninhos em cada temporada, pelo que a monitorização nas praias de nidificação continua a ser fundamental. No entanto, nas últimas décadas houve um aumento significativo da variedade de técnicas para o estudo da dispersão e ecologia trófica das tartarugas marinhas, que facilitam a compreensão dos mecanismos e factores que afectam a quantidade de fêmeas reprodutoras e a sua dispersão, permitindo delinear estratégias de conservação fundamentais, como a definição de unidades de gestão, corredores migratórios, e áreas de alimentação. Estas e outras questões fundamentais, tal como a composição, distribuição e dinâmica das populações, a sua conectividade, as estratégias reprodutoras, assim como as relações filogenéticas e filogeográficas entre populações e espécies beneficiaram ainda do uso crescente de marcadores genéticos e da sua optimização para as diferentes espécies. A abordagem genética permite descrever estruturas populacionais, permitindo definir unidades de gestão, o que tem particular importância para a conservação de determinadas populações. Especificamente, a utilização do DNA mitocondrial, um marcador de matrilinhagem, é útil para o estudo da estrutura genética entre populações reprodutivas e, por conseguinte, da fidelidade das fêmeas aos sítios de desova (filopatria). Através de comparações das relações entre linhagens de DNA mitocondrial e as suas respectivas áreas geográficas é possível inferir sobre a história populacional, colonização e dispersão a longa distância. Por outro lado, permite também, através da análise da composição genética de stocks mistos (*Mixed Stock Analysis*), inferir a origem materna de machos e fêmeas em vários estágios de vida, provenientes de habitats de alimentação, corredores migratórios e/ou oriundos de arrojamentos ou capturas incidentais. Os marcadores nucleares, de herança bi-parental, tais como os microssatélites, permitem quantificar o fluxo genético mediado pelos machos e fornecer, a partir da avaliação das variações nas suas taxas de mutação, informação

sobre processos populacionais a diferentes escalas temporais. Questões relacionadas com a ecologia das diferentes espécies, nomeadamente o uso de recursos disponíveis em áreas de alimentação, e a identificação destas áreas podem ser esclarecidas com a análise de isótopos estáveis, uma ferramenta que tem sido cada vez mais utilizada no estudo das interações tróficas e do uso de habitats por diferentes fases do ciclo de vida das tartarugas.

O arquipélago de São Tomé e Príncipe, localizado no Golfo da Guiné, na África Ocidental (Atlântico Oriental), abriga cinco espécies de tartarugas marinhas, sendo elas, ordenadas por abundância, a tartaruga verde (*Chelonia mydas*), oliva (*Lepidochelys olivacea*), de pente (*Eretmochelys imbricata*), de couro (*Dermochelys coriacea*), e comum (*Caretta caretta*). Acredita-se que estas ilhas possam ser também importantes para agregações de juvenis e sub-adultos de tartaruga verde e de pente que se alimentam nos seus recifes rochosos e pradarias marinhas. É neste arquipélago que se julga encontrar uma das populações de tartarugas de pente (*E. imbricata*) mais ameaçadas do mundo, a última população desta espécie no Atlântico Oriental, que se acredita ter sido severamente reduzida em resultado da exploração intensiva para o comércio de escamas. Os estudos anteriores sobre a diversidade genética de adultos desta espécie, assim como da tartaruga verde destacaram a sua alta distinção genética, alertando para a um grau elevado de vulnerabilidade destas populações na região. A tartaruga oliva (*L. olivacea*) tem sido sujeita a uma forte exploração para consumo da sua carne e ovos na ilha de São Tomé, mas pouco se sabe sobre esta população. O entendimento da dinâmica populacional dessas três espécies é essencial para o desenvolvimento de ações de conservação direcionadas e efetivas em diferentes escalas espaciais e temporais.

O objetivo geral desta tese foi investigar processos e mecanismos que afetam o atual estado de conservação das três principais espécies que ocorrem nas ilhas de São Tomé e Príncipe e levantar hipóteses sobre as perspectivas futuras dessas populações. Os objetivos específicos foram: (1) investigar a conectividade migratória das espécies na região e avaliar suas implicações para a resiliência da população no Atlântico Oriental; (2) contribuir para a compreensão da dinâmica populacional actual e passada, avaliando mudanças recentes no efectivo populacional e a capacidade potencial de recuperação; (3) compreender o comportamento de nidificação e alimentação e avaliar os padrões de distribuição temporal e espacial destes comportamentos.

A tese está organizada em cinco capítulos, sendo que os capítulos 1 e 5 são respectivamente uma introdução à biologia e ecologia das tartarugas marinhas e ao seu estudo, para enquadramento do trabalho realizado, e a discussão dos principais resultados obtidos. Os restantes capítulos são dedicados a cada uma das três espécies estudadas e incluem um ou mais artigos que correspondem a trabalhos já publicados ou submetidos para publicação em revistas com circulação internacional e sujeitas a revisão por pares: capítulo 2 – *Chelonia mydas* (3 artigos), capítulo 3 – *Lepidochelys olivacea* (um artigo) e capítulo 4 – *Eretmochelys imbricata* (um artigo).

No capítulo 2 explorei vários aspectos relativos à ecologia, dispersão e reprodução das tartarugas verdes do arquipélago. O artigo 1 comprova que as tartarugas verdes juvenis e adultas de São Tomé e Príncipe exibem altos níveis de diversidade genética e são geneticamente diferenciadas de outras populações de juvenis e adultos do Atlântico. Esta diversidade foi avaliada através da análise do $_{mt}$DNA, complementando um estudo anterior com a utilização de mais indivíduos, assim como de sequências mais longas (melhorando a sua resolução) e usando microssatélites pela primeira vez nestas populações. A análise de *stocks* mistos revelou a maioria dos juvenis estudados são recrutados ao nível da população reprodutora de São Tomé e Príncipe, o que sugere que as tartarugas verdes no arquipélago apresentam dispersão limitada e devem ser consideradas uma unidade de gestão única para a qual ações de conservação devem ser implementadas não apenas ao nível das fêmeas reprodutoras, mas também dos juvenis. No artigo 2 aprofundei a informação sobre os juvenis, ao identificar as principais áreas de alimentação na ilha de São Tomé e ao usar isótopos estáveis para compreender a sua ecologia alimentar. Os resultados mostraram que esta espécie demonstra uma plasticidade em termos de exploração de recursos existentes e que esta varia de acordo com a classe etária dos juvenis; indivíduos de tamanhos distintos segregam em diferentes habitats e ocupam nichos tróficos distintos que se mantêm durante longos períodos de pelo menos vários meses. Estes resultados mostraram que a ilha de São Tomé fornece uma variedade de importantes habitats de recrutamento / desenvolvimento para os seus juvenis. No artigo 3 explorei as limitações impostas pela monitorização incompleta dos locais de nidificação, ou por baixas taxas de recaptura devido à imprecisão da fidelidade das fêmeas ao local de nidificação, que comprometem a obtenção de dados robustos sobre a abundância e distribuição das populações nidificadoras em São Tomé e Príncipe. Para superar essas restrições, usei dados de marcação e recaptura para desenvolver um modelo focado no indivíduo que permitiu caracterizar estatisticamente o comportamento de nidificação das populações de tartarugas verdes e olivas,

usando uma abordagem inovadora para estimar o intervalo de tempo entre desovas, tendo em consideração diferentes fatores que levam à heterogeneidade observada na duração dos períodos entre desovas, incluindo a probabilidade de uma fêmea abortar um processo de nidificação.

No capítulo 3, dedicado à tartaruga oliva (*L. olivacea*), incluí o artigo 4, para o qual usei marcadores nucleares para caracterizar o comportamento reprodutor da população adulta (machos e fêmeas) desta espécie na ilha de São Tomé e o papel que este tem para a manutenção da diversidade genética desta espécie na região. Neste sentido, verifiquei que esta população reprodutora exibe poliandria, e que a proporção dos machos é superior à das fêmeas. Este comportamento tem vantagens, uma vez que identifiquei que a dispersão desta população é mediada pelos machos, o que potencia o fluxo genético entre São Tomé e Príncipe e outras zonas do Atlântico Oriental. No entanto, estas estratégias de história de vida parecem ser insuficientes para evitar a perda de diversidade genética como resultado de um efeito de gargalo, provavelmente relacionado com a exploração intensiva de fêmeas reprodutoras nas praias de nidificação de São Tomé nas últimas décadas.

No capítulo 4 (que inclui o artigo 5), apresento os resultados de um levantamento exaustivo das praias de nidificação da tartaruga de pente (*E. imbricata*) que possibilitou, através de modelação, obter a primeira estimativa do número total de actividades de reprodução por ilha e por praia, assim como uma estimativa do número de fêmeas que compõem esta população. A identificação das principais praias usadas por esta espécie foi complementada por um estudo sobre o nível de impacto humano e susceptibilidade das fêmeas a perturbações em cada praia, usando uma ferramenta de classificação de adequabilidade e de nível de ameaça para praias de desova. Com este estudo foi possível identificar o ilhéu das Rolas como o principal local de nidificação desta espécie em São Tomé e Príncipe; apesar desta espécie nidificar principalmente em praias relativamente isoladas, a maioria destas praias encontra-se sob pressão humana, que deverá ser mitigada dado o reduzido tamanho desta população, estimado em menos de 75 fêmeas reprodutoras.

Palavras-Chave: Recrutamento, dispersão, diversidade genética, ecologia alimentar, estratégias reprodutoras

ABSTRACT

Population declines of sea turtles worldwide, driven by overhunting, habitat loss, and other anthropogenic factors have made these animals a global conservation priority. Sea turtle species are particularly susceptible to anthropogenic and natural disturbances due to their complex life traits: female homing and migration, migrations of juveniles and males that remain poorly known, and a strong climatic influence on resources, breeding success and clucth sex-ratio. São Tomé and Príncipe archipelago in the Gulf of Guinea, West Africa, hosts at least four species of sea turtles, for three of which life-history traits, reproductive behavior and dispersal were assessed for this study: the green turtle (*Chelonia mydas*), the most abundant species, the hawksbill (*Eretmochelys imbricata*), which is considered the most threatened population in the Atlantic (both species common to both islands), and the olive ridley (*Lepidochelys olivacea)*, which only occurs in São Tomé island. In this study I integrated various tools and techniques, including site-based monitoring (e.g. on nesting beaches or foraging areas), genetic analyses for both adult and juvenile populations, mark-recapture studies, dispersal simulations as well as stable isotopes analysis, which complemented each other in the assessment of the conservation of each species in the archipelago, including little understood groups such as juveniles and males. Specifically, I showed that São Tomé island hosts important foraging areas that offer a variety of food sources for green turtle juveniles, which are recruited directly from this rookery. For the olive ridley turtle, I characterized the reproductive behavior of the adult population using paternal assessments and showed that males are important mediators of gene flow in this genetically depressed population. Finally, I conducted the first full characterization of spatial and temporal characterization of hawksbill nesting in the archipelago, identifying the key nesting habitats and assessing the levels of human impact that they are exposed to. Overall, the results of this study highlight the high vulnerability of the three species studied in light of limited dispersal, high genetic distinctiveness and exposure to threats.

Keywords: Recruitment, dispersal, genetic diversity, foraging ecology, reproductive behaviour

CHAPTER 1

GENERAL INTRODUCTION

“Sa ôdji di omali é cá depende d’inê, mage vida d’inê cá dêpendê di bô”
We depend on the oceans but the life in our oceans depends on us

- People of São Tomé and Príncipe

GENERAL INTRODUCTION

Several marine species undergo periodic long-distance migrations in search of optimal foraging conditions, safety, and reproductive opportunities (e.g., Limpus et al. 1992; Block et al. 2011; Hays & Scott, 2013; Cherry et al. 2013; Jaine et al. 2014). By taking advantage of resource peaks or avoiding periods of heightened mortality risk over time and space, these species may sustain considerably larger populations than otherwise similar resident species (Alestram et al. 2003; Bauer & Hoye, 2014). However, in light of the observed declines of several migratory species, conservation biologists have argued that long distance animal migrations are now an endangered pattern of the natural world (Harris et al. 2009; Wilcove & Wikelsky, 2008), a phenomenon that can potentially affect the structure and functioning of entire ecosystems (Lundberg & Moberg, 2003; Bauer & Hoye, 2014). Conserving migrant species poses major scientific and political challenges, and efforts are often hindered by the difficulty of studying animals that are constantly on the move. Historically, research on animal migration has focused on the migrants themselves: how, when, where, and why animals migrate (Alerstam et al. 2003), in an attempt to answer some of the challenges of migration biology (Bowelin et al. 2010). Even nowadays, for many iconic migratory large species, fundamental data on such basic topics as migratory routes, population structure, diet, size at establishment at foraging or reproductive grounds, among others, are still lacking.

Marine turtle migrations

Marine turtles are considered one of the most fascinating migratory group of animals, as they inhabit a variety of neritic and pelagic habitats, from the tropics to subarctic waters and venture onto terrestrial habitats to nest or bask in tropical and temperate latitudes, where they are easily accessible. There is a total of 7 species of marine turtle in the world, only two of which are endemic to a specific region: the Australian flatback (*Natator depressus*) and the small Kemp's ridley (*Lepidochelys kempii*), which inhabits the Gulf of Mexico. Of the remaining species, the green (*Chelonia mydas*) and hawksbill turtles (*Eretmochelys imbricata*) typically inhabit sub-tropical and tropical coastal regions, the olive ridley turtles (*Lepidochelys olivacea*) have a circumtropical distribution, the loggerhead (*Caretta caretta*) is found in sub-tropical and temperate waters, and the leatherback (*Dermochelys coriacea*) is widely distributed throughout the world's oceans from boreal to tropical waters (Fig. 1).

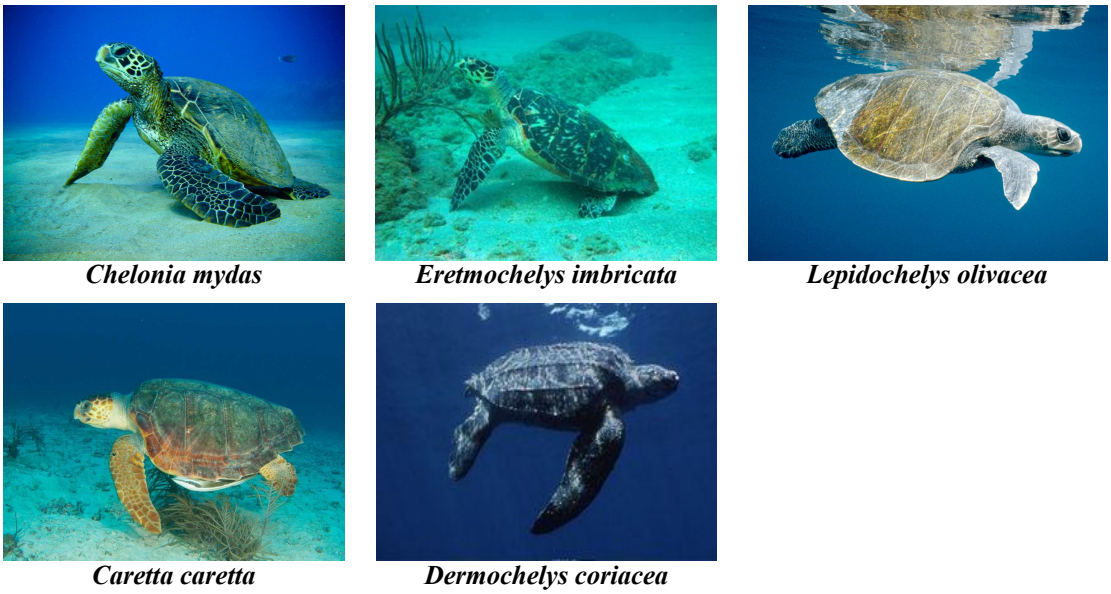

Figure 1. The five main species occurring in the Atlantic Ocean, including those on which this thesis is focused (top three)

Most species of marine turtles migrate intermittently throughout their lives. As hatchlings, a mixed strategy of directional swimming and passive drift leads them through more or less complex migratory pathways that sometimes cross entire ocean basins and back (Shillinger et al. 2008; Bowen et al. 1995; Bolten et al. 1998; Boyle et al. 2009), depending on the species. The biology of post-hatchling and early juvenile stages (usually referred to as the "lost years") is the least understood, and for most sea turtle species the location or duration of the early juvenile stage is still unknown. However it is generally accepted that there are three basic developmental life history patterns observed for marine turtles (Fig. 2): (1) complete development in the neritic zone (*N. depressus*); (2) early juvenile development in the oceanic zone and later juvenile development in the neritic zone (*C. mydas*, *E. imbricata*, *C. caretta* and some populations of *L. olivacea*) and, (3) complete development in the oceanic zone (*D. coriacea, L. kempii* and most populations of *L. olivacea*).

Species exhibiting either the Type 1 or Type 3 pattern commit to either the neritic or oceanic zone, respectively, for their entire developmental stages as well as for the adult foraging stage. Species with the Type 2 pattern have major habitat changes during their development, as they take up residence on successive neritic feeding grounds (Limpus & Musick, 2017), often showing fidelity to specific foraging areas, returning to them reliably after long, seasonal migrations or experimental displacements (Papi et al. 2000; Avens et al. 2003; Lohman et al. 2008; Schofield et al. 2010). Once adults, they may leave neritic habitats during the reproductive migrations, which may involve oceanic migration corridors between the adult

foraging areas (neritic) and internesting habitats (also neritic) (Bolten, 2003). These reproductive migrations may take place every 1, 2 or 3 years, depending on food availability at the foraging grounds, with male turtles thought to return to the foraging grounds soon after mating, and females remaining near the reproduction area for several weeks, during which they will lay several clutches at regular intervals (Fig. 3).

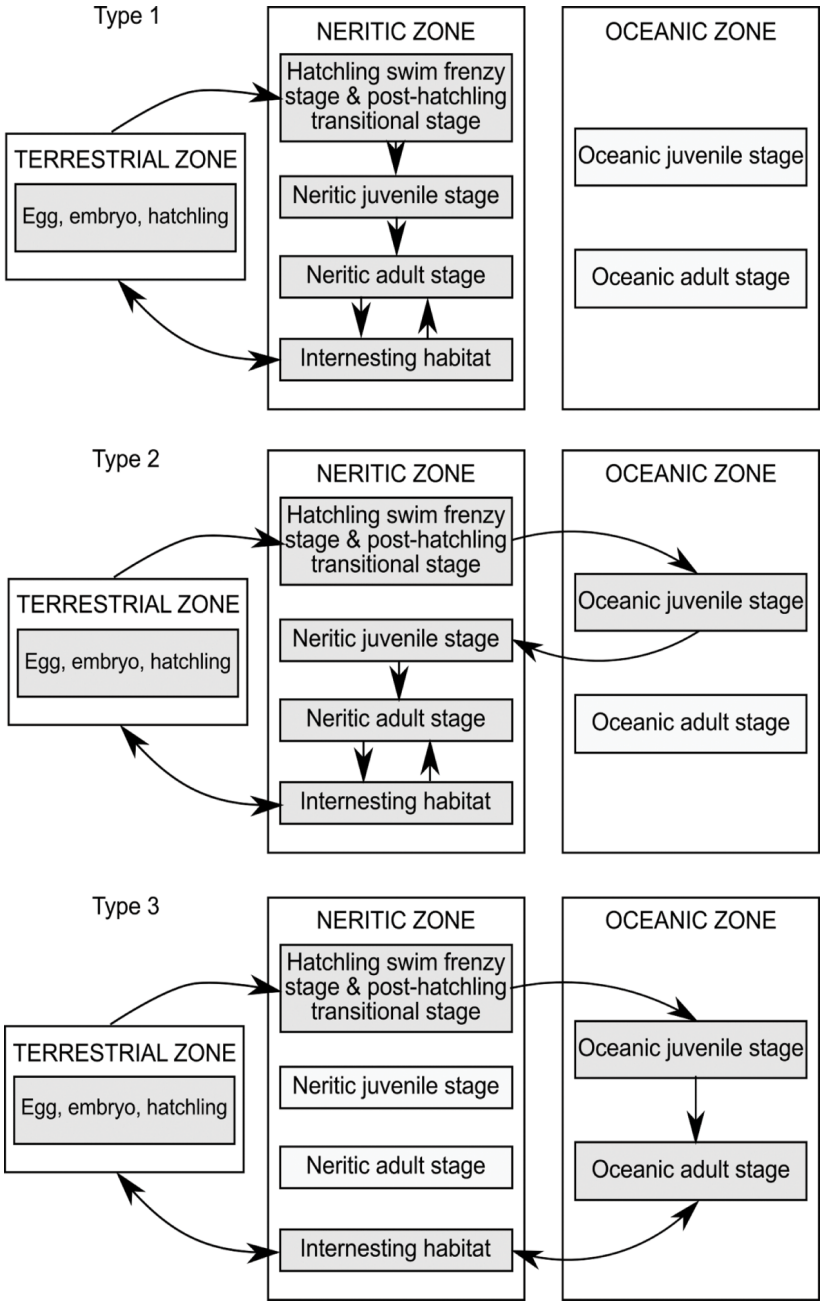

Figure 2. Three distinct sea turtle life history patterns illustrating the sequence of ecosystems inhabited (from Bolten, 2003).

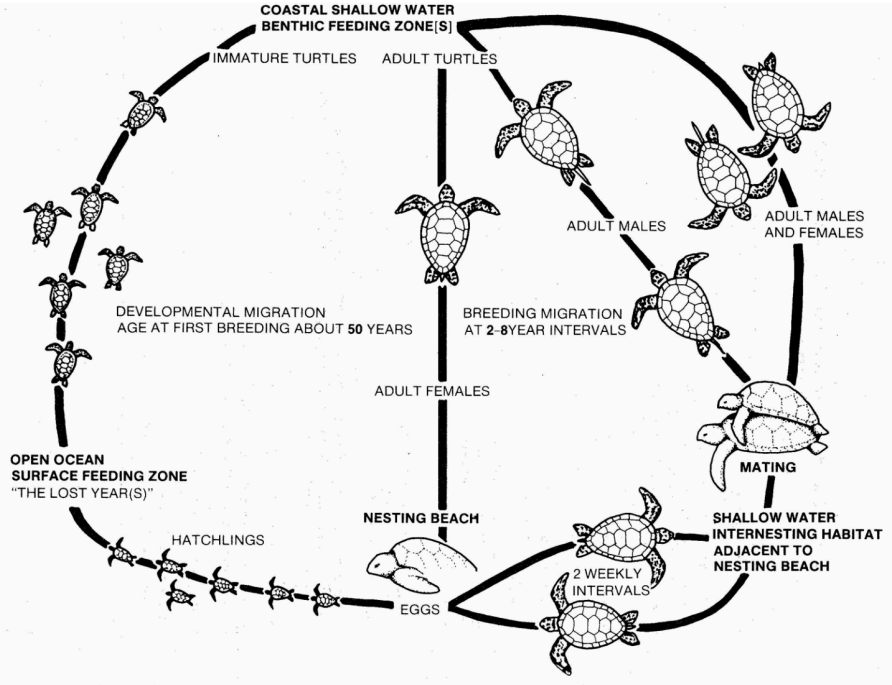

Figure 3. Schematic diagram of the generalized sea turtle life cycle, with each species exhibiting variations on this central theme (Adapted from Southwood & Avens, 2010)

Natal homing and colonization of new sites

Adult turtles are thought to nest near the same geographic area where they themselves emerged as hatchlings, after spending years in distant oceanic regions, in neritic foraging grounds, or both (Bowen et al. 1994; Fitzsimmons et al. 1997; Bowen & Karl, 2007; Lohman et al. 2013), a behavior pattern known as "natal homing". The natal homing hypothesis, now widely accepted, was first speculated by mark-recapture observations and early analysis of mitochondrial DNA ($_{mt}$DNA) structure in Atlantic green turtle populations that showed that geographically distant rookeries were found to have heterogeneous $_{mt}$DNA haplotype frequencies (Encalada et al. 1996), with similar observations done in the Australasian region (FitzSimmons et al. 1997). The mechanisms by which marine turtles navigate back and forth between these sites has been one of the great research issues in sea turtle biology (Lohman et al. 2008, 2013), and a recent study strongly supports the geomagnetic imprinting hypothesis, by which turtles imprint on the unique geomagnetic signature of their natal area and use this information to return (Brothers & Lohmann, 2015).

Upon arrival at their breeding grounds, the mechanisms by which nesting females choose a specific beach or site on a beach are poorly understood (Mortimer, 1995). Turtle species share broad nesting requirements, which include deep, relatively loose sand above high-tide level. In fact, there is inter- and intraspecific variation in preference in terms of more specific physical features of a beach, such as length, width, height, slope, orientation, and vegetation (Hays et al. 1995; Wood & Bjorndal., 2000; Kamel & Mrosovsky, 2004, 2006). While many of the world's marine turtle nesting sites are located along the coasts of tropical American, African and Australasian continents, there are a notable number of sites located in islands. Volcanic islands provide access to resources and landscapes that are not always readily available on the larger continental beaches, including seclusion, little disturbance, a variety of nesting habitats and lower predation. Moreover, islands often have superior marine resources in adjacent coastal shelves. It is not clear however, how marine turtles colonized these islands, which are often very remote and distant to major nesting sites located in continental coasts. Some light has been shed about how remote islands, such as Ascension Island, located in the south Atlantic, became one of the most important nesting colonies for the green turtle in the Atlantic. Tag returns and genetic studies indicate a remarkable migratory circuit between Ascension Island and Brazil, a link for which at least two hypotheses have been proposed and tested (Bowen et al. 1989). One, which suggests a gradualist scenario in which nesting turtles tracked a series of progressively distant volcanic islands, based on the fossil record that indicates that turtles of the family Cheloniidae inhabited the proto-Atlantic prior to the separation of Africa and South America, about 70 million years ago. As these island chains became gradually more distant from South America by the action of sea-floor spreading, nesting turtles may have developed a progressively longer migratory route, culminating in the contemporary migration to Ascension Island. The second hypothesis more generally accepted suggested a rare and possibly recent colonization event to explain the presence of the Ascension Island rookery. In fact, imperfect natal homing, resulting in occasional colonization of newly emerged nesting habitat, such as geologically recent volcanic islands, may have provided a flexibility in migratory behaviour that so far has prevented the extinction of marine turtle species.

Marine turtle occurrence in São Tomé and Príncipe archipelago

The islands of the Gulf of Guinea form part of a volcanic chain that originated from the middle to late Tertiary, situated on the oceanic sector of a straight axis, the Cameroon volcanic line, about 1500 to 1600 km long (Déruelle et al. 1991; Burke, 2001; Caldeira & Munhá, 2002). Of these islands, the continental-shelf island Bioko, situated approximately 32 km from Cameroon, is the largest and closest to the African mainland, to which it was formerly connected. The other three islands, São Tomé, Príncipe and Annobón, are truly oceanic; they are smaller than Bioko

and were never connected with the mainland or with each other. Príncipe is situated about 220 km southwest from Bioko and 146 km northeast from São Tomé and is the oldest; it has an area of approximately 128 km^2 and an estimated age of 31 million years. The island of São Tomé, located about 275 km westwards from Gabon, is larger (836 km^2) and significantly younger, being around 13 million years old. Annobón (17 km^2) is the youngest island with an age of about 4.9 million years (Lee et al. 1994). São Tomé island is quite unique since it is surrounded by shallow reef habitat, unusual along the west African coastline, and a gentle bathymetric gradient offshore, uncommon for a volcanic island, offering optimal conditions for the colonization of marine turtles, both on its beaches, but also on foraging sites.

Four species nest regularly on São Tomé and Príncipe, most notably the green (*C. mydas*) and the olive ridley (*L. olivacea*), with some lower density nesting of hawksbill (*E. imbricata*) and leatherback (*D. coriacea*), and the rocky reefs surrounding the islands hold foraging aggregations of green and hawksbill juveniles. Despite modern surveys of the Atlantic coast of Africa for marine turtles began as early as 1957 by Carr (1957), it was not until 1994 that the first attempts to identify nesting species and nesting beaches in São Tomé and Príncipe were conducted (Graff, 1996). As confirmed by recent surveys, nesting occurs on the northern, eastern and southern coasts of São Tomé. The northern and eastern coastal areas host the largest human settlements, while the southern (especially southwestern) beaches are relatively pristine. The apparent lack of nesting on the western coast is likely due to the rocky substrate of the beaches stretching the length of the coastline.

Little is known about the biology and ecology of marine turtles in São Tomé and Príncipe, but in general terms, nesting season in these islands is initiated after the first rains (September–November), peaks in November – January, and continues at low density until March, with hatching peaking after two months of the nesting peak. Recent population estimates presume that these islands harbour one of the last remaining hawksbill nesting aggregations in the Eastern Atlantic region, leading to some authors to consider this region one of the most threatened Regional Management Units for Marine Turtles (RMU's; Wallace et al. 2010). Genetic studies of green (Formia et al. 2006) and hawksbill (Monzón-Arguello et al. 2010) marine turtles nesting on this archipelago indicate clearly that these populations represent single management units due to their high distinctiveness and significant divergence in allele frequencies when compared to the other rookeries in the Atlantic, urging for aggressive conservation measures. This genetic distinctiveness has been hypothesized to result from the sea level drop during the Pleistocene glaciation events which may have increased the amount of suitable nesting habitat available in São Tomé island, thus supporting a large ancestral

population that persisted in this ice age refugium as far back as the mid-Tertiary (Formia et al. 2006). Those large sea turtle populations may have only recently declined to present levels as a result of overexploitation which started with the arrival of the first humans in the 1470s. This scenario, although having only been postulated for green turtles, is likely to be similar for the other species occurring on these islands.

Challenges in marine turtle conservation in São Tomé and Príncipe

The threats that marine turtles face in West Africa were reviewed by Formia et al. (2003), an assessment that remains accurate to this date. In São Tomé and Príncipe, a relatively undeveloped nation, the biggest threat is perhaps that, in this archipelago, as in many coastal nations, marine turtles are considered significant sources of food and income. Particularly in São Tomé island, over-harvesting of fish by the abusive use of illegal nets by small-scale fishermen resulted in a decrease in catch, which lead to a greater dependence on other resources, such as sea turtles. Until 2014, when national legislation decreed that marine turtle trade and consumption was illegal and subjected to heavy fines (Decreto-Lei 8/2014), marine turtle meat, eggs and other products were openly sold in the main markets. Moreover, São Tomé and Príncipe were historically the major tortoiseshell exporters in West Africa, a product that is used to make ornaments and souvenirs for sale to tourists and has led to the near extirpation of the local hawksbill population.

Indirect threats include incidental captures by commercial fisheries operating in the Gulf of Guinea, affecting mainly olive ridleys and leatherbacks (Riskas et al. 2013), oil exploitation, a major economic activity in the Gulf of Guinea region (Weszkalnys, 2009) and sand extraction for construction work, both on-site and off-shore, that has presumably led to the collapse of several beaches on São Tomé island, especially on the northern shore.

Figure 4. Some of the main threats identified on São Tomé island: (a) Sand mining in October 2018 in the city of São Tomé, (b) marine turtle meat and eggs for sale in São Tomé market, January 2016; (c) beach erosion on Jalé and Praia Grande beaches

Advances in marine turtle conservation biology

Studying migratory animals demands for an integrative research strategy; increasing research on different migratory species has led to a better understanding of the underlying patterns of migration and has provided general hypotheses about the ecology and physiology of migrating animals (Dingle & Drake, 2007). When coupled with research on individual reproductive success and survival, these tools can be used to understand how populations are regulated (Runge & Marra, 2005; Wilcove & Wikelski, 2008), and how migration itself evolves (Robinson et al. 2010).

However, studies about the movements of migratory animals are often limited by logistical and financial constraints, so the advent of the use of indirect methods, tools and indicators such as genetic markers or stable isotopes, coupled with behavioural or morphological measurements has proven to be useful in the study of marine turtle movements and in stock resolution.

Genetic markers We now know that marine turtle rookeries, being shaped by natal homing, are functionally independent management units (MU). As so, they exhibit distinct demographic processes leading to inter-population variation in life history traits and population dynamics that warrant population-specific management schemes (Wallace et al. 2010). Improving our ability to define and improve the resolution of management units has been possible due to, in part, the variety of molecular genetic analyses available, including the analysis of mitochondrial and nuclear DNA (microsatellites), often in combination. The variety in the use of molecular markers of sea turtles is reviewed in Jensen et al. (2013), highlighting the contribution of genetic tools towards evidencing regional natal homing by breeding adults, establishing connectivity between rookeries (i.e., nesting colonies) and foraging habitats, and defining phylogeography and broad scale stock structure for most species.

Mitochondrial DNA ($_{mt}$DNA) for instance is widely used since it is fast and easy to sequence, the principal non-coding region is highly polymorphic and, most important, it is maternally inherited. For these reasons it has been used for resolving nest site fidelity (Bowen et al. 1989), homing behaviour (e.g. Allard et al. 1994; Fitzsimmons et al. 1994; Encalada et al. 1996), phylogenetic relationships (Bowen et al. 1991, 1992), phylogeographic patters (e.g. Bowen et al. 1997a,b; Bowen & Kark, 2007), and to identify the maternal origin of both males and females of various life stages and at different foraging grounds (e.g. Lahanas et al. 1995; Bolten et al. 1998; Bowen et al. 2007; Proietti et al. 2009). Because this marker can have limited resolution, microsatellites (biparentally-inherited nuclear DNA, nDNA), which have many

alleles per locus, can be used in individual and familial genotyping, thus contributing towards the elucidation of small-scale population structure, and in parentage studies. Additionally, nDNA provides information about the reproductive biology of males and male-mediated gene flow. When supplemented with information on threats and the influence of environmental conditions on phenology and behaviour, these molecular tools facilitate robust definitions of MUs for marine turtles at multiple scales, helping to address different management and research challenges. Because the analysis of microsatellites is time and labour intensive, scaling up in projects requiring analyses across thousands of samples can be challenging. Advances in the application of genetics in marine turtle biology and conservation, including the use of other genetic markers such as single nucleotide polymorphisms (SNPs) and the advent of genomics have been explored and discussed recently by Komoroske et al. (2017).

Stable isotopes Obtaining meaningful dietary information from live, free-living turtles requires their capture and stomach lavages, a method that is quite invasive. Although useful, the analysis of gut contents can only provide limited information, as ingested food items need to be present and identifiable at the time of examination, while such data only yield a relatively proximate indication of dietary choice (Godley et al. 1998, Votier et al. 2003). The analysis of stable isotopes assimilated by a consumer and its comparison with the isotope ratios of different diet items helps to overcome these limitations, as in particular the ratios of the stable isotopes of nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$) and carbon ($^{13}C/^{12}C$ expressed as $\delta^{13}C$) in the consumer tissues tend to reflect those of the diets in a predictable way (DeNiro & Epstein, 1981; Peterson & Fry 1987). The $\delta^{15}N$ signatures show a stepwise enrichment at each successive trophic level within a food chain (Hobson et al. 1994; Adams & Sterner, 2000), while $\delta^{13}C$ values can provide information about the source of carbon at the base of a food chain, since the rate at which they are fixed in plants differ among photosynthetic pathways (Vogel, 1993). This is particularly important in the marine ecosystems, since phytoplankton have lighter $\delta^{13}C$ than many inshore plants, and thus Carbon isotopes can be useful to distinguish inshore vs. offshore feeding sources (Fry & Sherr, 1984; Hobson et al. 1994). This information can also be enriched by analyzing the ratio of sulphur isotopes ($\delta^{34}S$), which also varies substantially among primary producers, and are often distinct in benthic and pelagic marine waters (Connoly et al. 2004). Stable isotope analysis has yielded important and novel insights into intra and inter-species trophic relationships and into trophic interactions on both spatial and temporal scales (Hobson et al. 1994; Chouvelon et al. 2012). Moreover, within an organism, different tissues incorporate stable isotopes at different rates; a fine example are the sea turtles, as epidermis and keratin have longer assimilation periods, thus reflect long-term foraging history, while red blood cells

reflect a recent diet (Seminoff et al. 2006, 2009; Reich et al. 2008). Therefore, such tissues collected from turtles at breeding areas reflect their dietary history at foraging grounds prior to migration to the breeding area (e.g. Hatase et al. 2010, Zbinden et al. 2011, Pajuelo et al. 2012), and may be used to study ontogenetic diet or habitat shifts (Reich et al. 2007; Arthur et al. 2008; Snover et al. 2010; Velez-Rubio et al. 2016). Dual-isotope multiple-source mixing models have been developed to quantify the proportions of various prey in the diet of several marine organisms and have been applied successfully to sea turtles (Dodge et al. 2011; Lemons et al. 2011; Shimada et al. 2014).

Population modelling Assessments of sea turtle populations are based primarily on the census of nesting females at specific beaches. The number of turtles reproducing on a given season is highly dependent on the acquisition of energy reserves not only to undertake often extensive migrations from the foraging areas to the nesting sites, but to invest in vitellogenesis (egg production). If these energy requirements are not met, nesting will be delayed until feeding conditions improve (Solow et al. 2002; Rivalan et al. 2005) or can be reflected in reproductive investment (Saba et al. 2007). For this reason, several reproductive parameters, such as clutch size, clutch frequency and remigration intervals show intra and inter population variation (Solow et al. 2002; Wallace et al. 2006). Furthermore, phenotypic heterogeneity can mask or exacerbate individual allocation patterns when trends are averaged across a population, which is problematic in the case of sea turtles (Hays, 2000). Finally, differential detectability of nesting females or poor sampling regimes may affect population estimates (Beissinger & Westphal, 1998; Holmes, 2001). Modelling of key reproductive parameters for a given population sheds some light on similarities and differences in life-history characteristics across body sizes, populations, species, and phylogenetic groups, and are important in exploring correlations between characters such as fecundity and age at maturity, age at maturity and adult lifespan, and so on, parameters which are key to estimation of population size and for population viability analyses (Stearns, 1992; Heppel, 1998). Population modelling has been used in sea turtle research to assess population viability and the risk of harvesting for long-term stock viability (e.g. Chaloupka, 2002; Mazaris et al. 2005), effects of age dependent mortality (Mazaris et al. 2006), growth rates (Chaloupka & Limpus, 1997), population trends (e.g. Richardson et al. 2006) and demographic parameters (e.g. Prince & Chaloupka, 2012).

THESIS AIMS AND OUTLINE

The work done in this thesis aimed to shed some light into unknown aspects of the life of these species in the region, particularly concerning less studied life stages such as males and at-sea juveniles, to explore their connectivity with other populations in the Atlantic, and to evaluate how their reproductive and foraging behaviour may affect their resilience to threats. I also aimed to obtain for the first time baseline information that can in the future be used to establish trends and assess population changes over time. To achieve this, I combined several methodologies, including genetic and stable isotope analyses, and modelling of individual reproductive behaviour.

This thesis is structured in three main chapters, each one dedicated to one of the three species studied. In a final chapter I present a general discussion of the main results of the thesis which highlight the importance of the populations of these species in São Tomé given low dispersal, genetic distinctiveness and levels of threat specific to this archipelago.

Chapter 2 – *Chelonia mydas*

This chapter is dedicated to the green turtle (*Chelonia mydas*) and includes three papers corresponding each to a specific line of research developed to answer the following specific questions:

Paper 1 What are the current levels of genetic diversity of *C. mydas* and how can they be related to the dispersal and recruitment of this species in São Tomé and Príncipe archipelago;

Hancock, J. M., Vieira, S., Taraveira, L., Santos, A., Schmitt, V., Semedo, A., Ferrand, N., Gonçalves, H. & Sequeira, F. (2019). Genetic characterization of green turtles (*Chelonia mydas*) from São Tomé and Príncipe: Insights on species recruitment and dispersal in the Gulf of Guinea. *Journal of Experimental Marine Biology and Ecology*, *518*, 151181.

Paper 2 How are juvenile sea turtles of different life-stage groups distributed in São Tomé island, which trophic niches do they occupy, and which is the relevance of the S. Tomé island coast as a foraging and settling ground for juveniles:

Hancock, J. M., Vieira, S., Jimenez, V., Rio, J. C., & Rebelo, R. (2018). Stable isotopes reveal dietary differences and site fidelity in juvenile green turtles foraging around São Tomé Island, West Central Africa. *Marine Ecology Progress Series*, *600*, 165-177.

Paper 3 How to overcome field monitoring restraints in estimating the internesting period and the rank of an observed nesting event, two of the parameters that are necessary for the correct estimation of the number of nesting females on a given site:

Hancock, J.M., Vieira, S., Lima, H., Schmitt, V., Pereira, J., Rebelo, R., Girondot, M. (2019). Overcoming field monitoring restraints in estimating marine turtle internesting period by modelling individual nesting behaviour using capture-mark-recapture data. *Ecological Modelling, 402,* 76-84.

Chapter 3 - *Lepidochelys olivacea*

Paper 4 How are the reproductive behaviour and dispersal patterns of the olive ridley turtle (*Lepidochelys olivacea*) characterized in São Tomé and Príncipe and what are the implications for the maintenance of genetic diversity of this species in the region:

Hancock, J.M., Vieira, S., Lima, H., Rebelo, R., Ferrand, N., Sequeira, F., Gonçalves, H. Genetic diversity, multiple paternity and dispersal in an olive ridley (*Lepidochelys olivacea*) rookery from São Tomé island, West Africa (*in preparation*)

Chapter 4 – *Eretmochelys imbricata*

Paper 5 What are the key temporal and spatial patterns of the nesting of this species, and what are the implications of specific nesting site selection in terms of exposure to human impacts and beach suitability.

Hancock, J.M., Vieira, S., Lima, H., Besugo, A., Schmitt, V., Carvalho, H., Girondot, M., Rebelo, R. Reproductive biology and conservation status of the critically endangered Hawksbill sea turtle on São Tomé and Príncipe (*in preparation*)

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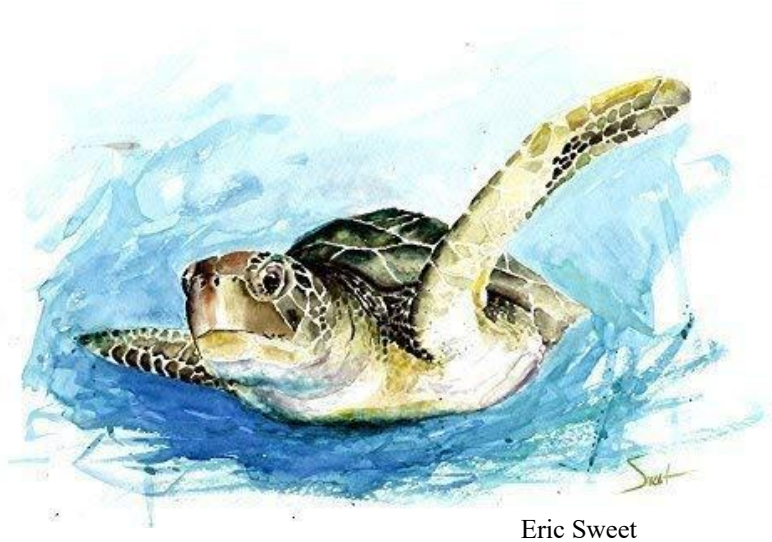
CHAPTER 2

TARTARUGA MÃO BRANCA

GREEN TURTLE



Eric Sweet

Chelonia mydas

PAPER 1

Genetic characterization of green turtles (*Chelonia mydas*) from São Tomé and Príncipe: insights on species recruitment and dispersal in the Gulf of Guinea

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"Human beings can learn valuable lessons in conservation of necessary personal resources for accomplishing the fundamental tenants of life by observing a judiciously paced turtle determinedly and stealthily traversing the world."

- Kilroy J. Oldster, Dead Toad Scrolls

Hancock, J.M., Vieira, S., Taraveira, L., Santos, A., Schmitt, V., Semedo, A., Patrício, R., Ferrand, N., Gonçalves, H., Sequeira, F. (2019). Genetic characterization of green turtles (*Chelonia mydas*) from São Tomé and Príncipe: Insights on species recruitment and dispersal in the Gulf of Guinea. *Journal of Experimental Marine Biology and Ecology*, *518*, 151181.

Genetic characterization of green turtles (*Chelonia mydas*) from São Tomé and Príncipe: insights on species recruitment and dispersal in the Gulf of Guinea

ABSTRACT

Genetic studies on green sea turtles (*Chelonia mydas*) in the Eastern Atlantic have mostly focused on reproductive females, with limited information available regarding juveniles and foraging grounds. Improved understanding of genetic diversity and patterns of connectivity between nesting and foraging grounds is critical to identify management units and delineate suitable conservation strategies. Here we analyzed data from 11 microsatellite markers and sequences of the mitochondrial control region from both juveniles and females sampled in foraging and nesting aggregations around São Tomé and Príncipe islands, in the Gulf of Guinea, West Africa. The analysis of mitochondrial markers show that São Tomé and Príncipe's juvenile and adult green turtles are genetically differentiated from other foraging and nesting Atlantic populations. Moreover, both nuclear and $_{mt}$DNA data were congruent in showing exhibit high levels of genetic diversity. The similar levels of genetic diversity found in both juveniles and females are consistent with the results from mixed stock analyses, which suggested that São Tomé and Príncipe's rookery is the primary source of juveniles to the local foraging areas. Taken these aspects in consideration, we argue that São Tomé and Príncipe green turtles show limited dispersal and should be considered an important management unit, and conservation actions in this archipelago must be implemented not only at the level of the rookery but should also include the foraging aggregations.

Keywords: *Chelonia mydas*; dispersal; connectivity; genetic diversity; Eastern Atlantic; Mixed Stock Analysis

INTRODUCTION

For highly migratory species, their ability to disperse affects the connectivity among populations, recruitment patterns, links between foraging and breeding areas and genetic diversity (Bowler et al. 2005; Blumenthal et al. 2009; Runge et al. 2015). In marine environments the patterns of dispersal and recruitment result from complex processes, often influenced by species-specific responses to features of both nearshore and pelagic environments. Marine turtles represent well this complexity, and are well suited to study these processes, since they have a complex life cycle that includes multiple phases within neritic and pelagic habitats (Van Buskirk & Crowder, 1994; McClellan & Read, 2007; Arthur et al. 2008) between which both juveniles and adults disperse widely over vast expanses of ocean. Post-hatchling dispersal from natal beaches is an interplay between oriented swimming and passive drift (Scott et al. 2012) and is followed by an epipelagic phase that ranges from five to ten years, after which they recruit to coastal foraging grounds as juveniles (Bolten, 2003). Recruits to a foraging ground can be an uneven mix of juveniles originating from rookeries located either in the vicinity or thousands of kilometers away (Bolten et al. 1998; Monzón-Argüello et al. 2010), originating aggregations of mixed genetic origins (Bowen et al. 2007). Factors influencing dispersal and settlement of juvenile sea turtles include passive drift in oceanic currents (Carreras et al. 2006; Okuyama et al. 2011), distance to the contributing nesting colonies (Lahanas et al. 1998), as well as water temperature and food availability (Mansfield et al. 2014). Upon reaching sexual maturity, adults periodically migrate between foraging and reproductive grounds showing a remarkable fidelity to specific areas for reproduction (Limpus et al. 1992; Lohmann et al. 2008), typically located in the vicinity of their natal beach (Bowen & Karl, 2007). This so called “natal homing” behaviour” leads to distinct breeding populations that, over time, may accumulate genetic differences that can be used as “genetic signatures” to identify groups of individuals back to their population of origin (Allard et al. 1994; Bowen et al. 1992; Encalada et al. 1996). Molecular markers have been an increasingly useful tool to understand patterns of connectivity and dispersal among populations of marine turtles, particularly through estimates of potential contribution of donor (rookeries) to recipient populations (mixed foraging grounds) using mixed-stock analyses (Pella & Masuda, 2001). Based on some of these studies, the high level of genetic substructure found among marine turtle nesting populations and foraging aggregations, even at a relatively small spatial scale, has led to the emergence of the management unit concept as a novel framework for prioritizing protection at a local/regional level (Wallace et al. 2010). Indeed, the increase in genetic studies have been crucial in improving indirectly our knowledge about behaviour, ecology and evolution of these species,

providing thus an important support for conservation and management (Bowen & Karl, 2007; Komoroske et al. 2017).

The green turtle (*Chelonia mydas*) is a highly migratory marine organism with a circumglobal distribution, occurring throughout tropical and subtropical regions and, to a lesser extent, temperate waters (Seminoff et al. 2015). This species is considered globally endangered (IUCN, 20018), as like other marine turtle species, it has been facing several threats related to the degradation and loss of nesting and feeding habitats, and especially with their over-exploitation both for food (meat and eggs) and for ornaments (shell) (e.g. Parsons, 1962; Early-Capistrán et al. 2018). This situation together with accumulated evidences for extensive population declines in different areas of the globe, have emphasized the importance of adopting conservation actions to protect marine turtle rookeries and feeding habitats (Wallace et al. 2011). Within the Eastern and South Atlantic basins, some comprehensive studies on green turtle populations using mitochondrial DNA ($_{mt}$DNA) sequencing have identified several genetically distinct rookeries, including those found in Poilão (Guinea Bissau), São Tomé and Bioko (Gulf of Guinea) and Ascension islands, which may potentially represent independent management units (Formia et al. 2006, 2007; Patrício et al. 2017a). These studies have also expanded the knowledge on migration patterns and connectivity among green turtle populations. For example, a recent genetic study on juvenile dispersal from Guinea Bissau (Patrício et al. 2017a), suggested a high connectivity between rookeries and juvenile aggregations within West African populations. Despite accumulated information on green turtle biology, most genetic studies have been so far based on the analysis of a single type of marker ($_{mt}$DNA sequences), and focused on rookeries, with limited information regarding juvenile aggregations and males.

The São Tomé and Príncipe archipelago is part of a chain of extinct volcanoes called Cameroon Line. Its two main islands, São Tomé and Príncipe, are true oceanic islands separated from the African continent by an ocean approximately 1800 m deep and located about 160 Km apart. These islands' shallow coastal shelf (less than 200 m wide) has been recently depicted as holding relatively important aggregations of foraging juvenile green turtles in the Gulf of Guinea (Hancock et al. 2018). The aim of our study is to characterize the genetic diversity of green turtles from São Tomé and Príncipe archipelago using a combination of genetic markers (a mitochondrial fragment and a set of microsatellites). More specifically, we will use $_{mt}$DNA information for i) estimating the contribution of different rookeries in the Atlantic to São Tomé and Príncipe mixed stocks to ascertain their origin (foraging ground-centric approach), and ii) determine the possible dispersal patterns between São Tomé and Príncipe rookeries to foraging

areas in the Atlantic (rookery-centric approach). Additionally, we will explore potential cryptic inter and intra-population structure by assessing the genetic diversity at both $_{mt}$DNA and microsatellite levels in the archipelago, providing thus the first assessment of this type of complementary molecular information for any green turtle population in West Africa. We will contrast levels of genetic diversity found in São Tomé and Príncipe islands with others previously documented Atlantic populations in order to evaluate the potential importance of these two islands for global green turtle conservation, especially at a regional level through delineation of functional units of management for conservation.

MATERIALS AND METHODS

Sample collection

Skin samples from 112 adult females were collected at the primary nesting beaches in the islands of São Tomé (n = 93) and Príncipe (n = 19), between October and February of 2015 and 2016 during night patrols conducted by the staff of Programa Tatô and Príncipe Trust, respectively. Foraging juveniles were hand-captured in both island of São Tomé (n = 34) and Príncipe (n = 7). Samples were taken from the trailing edge of the left hind flippers and stored in 96% ethanol. Sampling locations are detailed in Fig. 1.

Markers and laboratory procedures

Whole-genomic DNA was extracted from all samples collected using QIA Quick DNEasy columns (Qiagen, Inc., Valencia, CA, USA) following standard DNA extraction protocols. Sequences of a fragment (860 bp) of the mitochondrial Control Region (CR) and eleven microsatellite loci were chosen for analysis.

Microsatellites We used seven microsatellite loci previously developed for *Caretta caretta* (Cc5H07, CcP2F11, CCP7C06, Ccp7D04, Cc1F01, Cc5C08, Cc1G02, Shamblin et al. 2009), and seven for *Eretmochelys imbricata* (EIM09, EIM40, ERIM25, ERIM03, ERIM19, ERIM21, ERIM22; Miro-Herrans et al. 2008, Shamblin et al. 2013). Microsatellite amplifications were conducted in a Biorad T100 thermocycler using a Multiplex PCR Kit (QIAGEN) following manufacturer's instructions. The eleven microsatellite loci were tested and amplified separately and then combined in two multiplex reactions for the final amplification using the MULTIPLEX MANAGER v.1.2 software (Table S1, Supporting

Information). General thermal conditions comprised an initial denaturation for 15 min at 95°C, followed by an additional step at 95°C for 30 sec., followed by 21 cycles of 1 min 30 sec. duration, each at 60°C with -0.5ºC decrease per cycle (to ensure an optimal annealing temperature for each primer). A second round of equal number of cycles was programmed at a lower, constant temperature (50ºC), set for 1 min each, to exponentially increase the number of amplified fragments. A final extension at 60°C was programmed for 35 min to promote adenylation and to avoid -A peaks during genotyping. Polymerase chain reaction (PCR) products were separated by capillary electrophoresis on an automatic sequencer ABI3130*xl* Genetic Analyzer (AB Applied Biosystems). Fragments were scored against the GeneScan-500 LIZ Size Standard using the GENEMAPPER v.4.1 (Applied Biosystems) and manually checked twice.

mtDNA For PCR amplification and sequencing of the CR fragment, we used the primers LCM15382/H950 developed by Abreu-Grobois et al. (2006). Thermal conditions for amplifications consisted of 15 min at 95°C, followed by 40 cycles of 30 sec duration each at 56°C, 45 sec at 72ºC with a final extension at 60°C for 20 min. Successful amplifications were enzymatically purified, and sequenced following the BigDye Terminator v.3.1 Cycle sequencing protocol (Applied Biosystems). Sequencing products were separated in the same automatic sequencer ABI3130*xl* Genetic Analyzer, and were aligned and compared in the software SEQSCAPE 3.0 (Applied Biosystems)

Genetic diversity and population structure

Microsatellites The presence/absence of large allele dropouts and null alleles was determined using the software MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004). Departures from Hardy–Weinberg expectations (HWE) and linkage disequilibrium (LD) among the 14 loci were tested in GenALEx 6.503 (Peakall & Smouse, 2012), using the Markov Chain method (Rousset, 2008), and the respective significance was adjusted with sequential Bonferroni correction (Rice, 1989). Mean number of alleles (Na), allelic richness (AR) and average observed (Ho) and expected (He) heterozygosities over loci were estimated using GenALEx. We estimated the level of genetic differentiation (F_{st}, Weir & Cockerham, 1984) between São Tomé and Príncipe's populations using GenALEx. Statistical significance of F_{st} values was tested using 1000 iterations, and 95% bootstrapped confidence intervals (CI) were used.

mtDNA Standard summary statistics, including the number of haplotypes (H), haplotype diversity (*h*), and nucleotide diversity (π) were calculated in the software DNASP v.5.0 (Librado & Rozas, 2009). All detected haplotypes were aligned and assigned to published haplotypes in the Mitochondrial Sequence Database for the Atlantic Ocean green turtle, hosted by the Archie Carr Center for Sea Turtle Research (University of Florida, Gainsville, Florida, USA). This database together with available data compiled recently by Patrício et al. (2017a), were used to compare levels of genetic diversity and differentiation of São Tomé and Príncipe's population (STP) with other known Atlantic rookeries and foraging aggregations. For this analysis, we used a truncated fragment of 490 bp length, which has been historically used to gather genetic information of other Atlantic populations. We generated a dataset that included haplotype frequency data for São Tomé and Príncipe and existing data for 13 rookeries (n = 1927) and 18 foraging aggregations (n = 1789) in the Atlantic. The genealogical relationships among São Tomé and Príncipe haplotypes and haplotypes from other rookeries were inferred using a median-joining network analysis (Bandelt et al. 1999) implemented in the program POPART (Leigh and Bryant, 2015). Genetic differentiation between rookeries and foraging aggregations in the Atlantic was estimated in ARLEQUIN 3.5 (Excoffier & Lischer, 2010) through pairwise fixation indices (F_{st}) using haplotype frequencies. The interpopulation migration rates were estimated using the formula $F_{st} = 1/(4\ N_m + 1)$ (N_m as virtual number of migrants, Wright, 1978).

Historical demography of the adult (females) population was examined by the neutrality tests of Tajima's D (Tajima, 1989), Fu's Fs (Fu, 1997), and R_2 (Ramos-Onsins & Rozas, 2002), which evaluate whether the polymorphism conforms to a neutral model of evolution. Statistical significance was determined by comparing estimated values against a distribution generated from 10,000 random samples under the hypothesis of selective neutrality and population equilibrium, with no recombination (Hudson, 1990), using the coalescent simulator in DnaSP. Historical demographic changes were also examined by estimating fluctuations in the effective population size over time using the Bayesian Skyline Plot (BSP) method (Drummond et al. 2005), as implemented in BEAST 2.5.0 (Bouckaert et al. 2014). For this analysis, we used a strict clock model and a substitution rate of 0.0015×10^{-9} mutations/site/year (Lahanas et al. 1994). A MCMC sampling algorithm was used in the HKY model estimated by JMODELTEST 0.1.1 (Posada, 2008). The MCMC chains were run 200 million generations, sampled every 10,000 generations with a 10% burn-in. All results were examined using TRACER 1.6 (Rambaut et al. 2014). Convergence was assessed with ESS (effective sample size) >200.

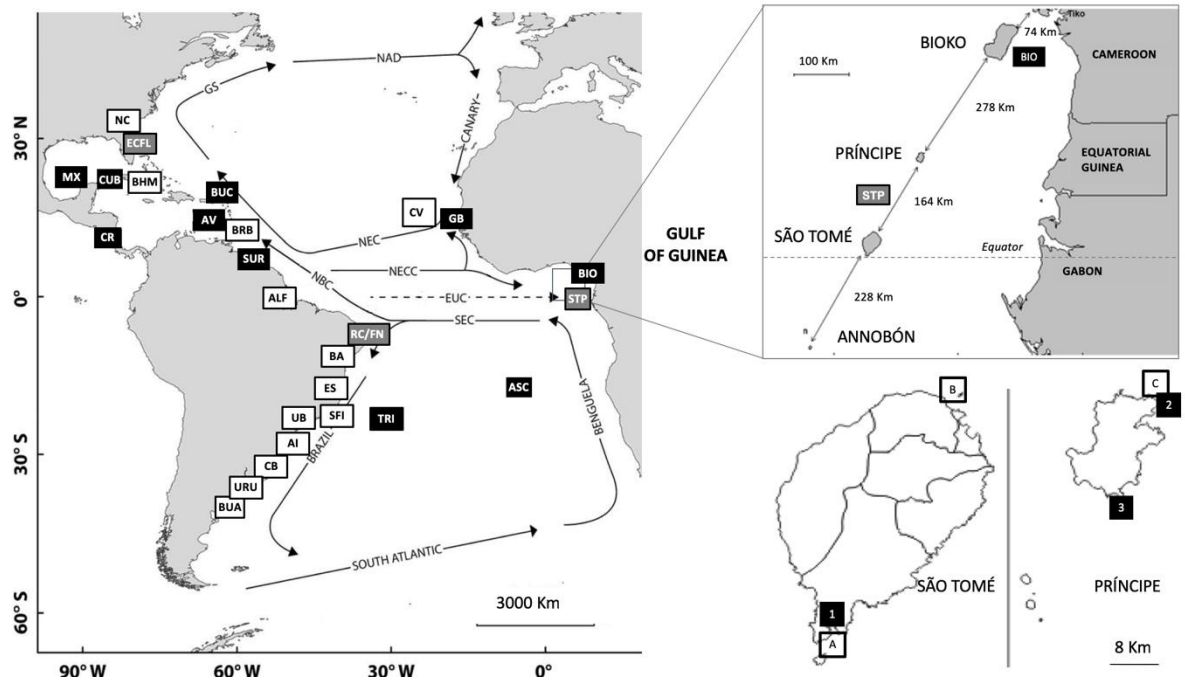

Figure 1. Location of green turtle (*Chelonia mydas*) rookeries (□), foraging aggregations (□) and locations where rookeries and foraging aggregations co-occur (■) included in this study and prevailing ocean currents (modified from Patrício et al. 2017a). Detailed location of São Tomé and Príncipe nesting sites and the three foraging areas sampled for this study are depicted in the figure inlet (Nesting areas: 1 – Porto Alegre; 2 – Praia Grande; 3 – Praia Grande do Infante. Foraging sites: A – Porto Alegre; B –Cabras islet; C – Mosteiros). Acronym list and references are included in Tables 1 and 2.

Mixed-Stock Analysis

A many-to-many Mixed-Stock Analysis (MSA) was performed to estimate the contributions of 14 green turtle stocks of the Atlantic to the São Tomé foraging grounds (foraging ground-centric MSA), as well as the dispersal of hatchlings originating from the São Tomé and Príncipe rookery (rookery-centric MSA). We used the ‘‘mixstock’’ package (Bolker et al. 2007) in R (R Development Core Team, 2011), a Bayesian algorithm that uses the MCMC method and a hierarchical model (Bolker et al. 2007), and WinBugs (Lunn et al. 2000) using rookery size as prior (Prosdocimi et al. 2012). São Tomé and Príncipe rookeries were grouped due to the lack of genetic differentiation (see results section) and geographic proximity (160 Km). Haplotypes observed by Formia et al. (2006) for the São Tomé rookery and their frequencies were added to our sample for this island (n = 26 females added), for a total sample size of 138 adult females used for this study. Because many of the previous studies used shorter sequence fragments (~490 bp), for comparative purposes, we used an initial mixed stock analysis (MSA) using

cropped sequences. Seven chains were run using 20,000 MCMC steps with a burn-in of 10,000 to calculate the posterior distribution. Convergence of MCMC estimates to a desired posterior probability was assessed using the Gelman–Rubin shrink factor (Gelman and Rubin, 1992), increasing the MCMC steps until all values obtained were less than 1.2. This diagnostic compares the variation of a single chain to the total variation among chains, and convergence is achieved if the shrink factor is less than 1.2 for each chain (Pella and Masuda, 2001). The final MSA was run twice, once with uniform priors (each rookery was equally likely to contribute with individuals to the São Tomé and Príncipe's foraging aggregations), and then using weighed priors (rookery size entered as a prior under the assumption that larger rookeries provide larger contributions to foraging grounds). The estimated sizes of each rookery were taken from Seminoff et al. (2015). Individuals with orphan haplotypes (i.e., not observed in any of the nesting rookeries) were removed from the analysis (Pella & Masuda, 2001). After we obtained our results from the MSA, we added these individuals back into the analysis and calculated the contribution of the 'unknown' rookeries to the stock mixture.

RESULTS

Genetic Diversity

Microsatellites All females and 30 of the 43 juveniles sampled were successfully genotyped at 14 microsatellite loci. Evidence of potential allele dropouts neither null alleles was found for the loci CCP7C06, ERIM19 and ERIM22, and for this reason these were eliminated from further analysis. Allele frequencies at all remaining 11 loci were within expectations of Hardy-Weinberg equilibrium, and no pairs of loci showed significant linkage disequilibrium after sequential Bonferroni correction. In the adult population, the number of alleles per locus ranged from 5 to 21, with an average of 11.5. The mean number of alleles ranged from 6.3 (São Tomé) to 6.8 (Príncipe). Levels of allelic diversity adjusted for sample size (allelic richness) ranged from 12.9 (São Tomé) to 10.0 (Príncipe), whereas observed and expected heterozygosity ranged from 0.804 ± 0.035 (São Tomé) to 0.799 ± 0.042 (Príncipe) and from 0.799 ± 0.037 (São Tomé) to 0.797 ± 0.037 (Príncipe), respectively. Regarding foraging grounds (juveniles), the mean number of alleles ranged from 6.5 (São Tomé) to 5.2 (Príncipe). Observed and expected heterozygosity ranged from 0.778 ± 0.040 (São Tomé) to 0.783 ± 0.029 (Príncipe) and from 0.811 ± 0.028 (São Tomé) to 0.790 ± 0.019 (Príncipe), respectively. Genetic diversity at the microsatellite level for females and juveniles sampled in São Tomé and Príncipe are presented in Table 1; comparisons with published data from other populations in the Atlantic are summarized in Table S2.

Mitochondrial DNA Sequencing alignment revealed 8 haplotypes in the sampled adult population, totalizing in combination with data from Formia et al. (2006), 9 distinct haplotypes (Table 2), and 7 haplotypes in the juveniles sampled at the foraging aggregations; summary statistics for resulting genetic diversity for each population are included in Table 2. The genealogical relationships among São Tomé and Príncipe female haplotypes together with available published data from other rookeries are depicted in Fig. 2. The haplotypes CM-A10, CM-A40 and CM-A75 are reported in these islands for the first time, while all other haplotypes observed in females (CM-A6, CM-A8, CM-A36, CM-A37, CM-A38) and in juveniles (CM-A5, CM-A8, CM-A10, CM-A36, CM-A40 and CM-A75) were already reported in Formia et al. (2006). The haplotype CM-A35, previously observed by Formia et al. (2006) in the nesting population, was not reported in this study, but was found in our sample of juveniles. The haplotype CM-A10 was found exclusively in the foraging aggregation, although it had been previously described for Ascension Island (Encalada et al. 1996; Formia et al. 2007) and Brazilian rookeries (Encalada et al. 1996; Bjorndal et al. 2006). There was one predominant haplotype, CM-A8, found in 57.6% and 65.1% of the samples (rookery and foraging aggregations, respectively). Overall, the haplotype diversity found in females was high (0.610 ±0.046) when compared to the $_{mt}$DNA diversity found in green turtle rookeries from the Eastern Atlantic and Ascension Island (Table S3).

Table 1. Levels of genetic diversity found in *Chelonia mydas* in São Tomé and Príncipe archipelago and individual islands, in both rookeries and foraging grounds, based on 11 microsatellite loci.

Population	N	Na (± se)	AR (± se)	Ho (± se)	He (± se)
ROOKERY					
São Tomé	72	12.9 (±1.36)	10.97 (±4.01)	0.803 (±0.035)	0.799 (±0.037)
Príncipe	**19**	10.0 (±1.07)	10.26 (±4.01)	0.799 (±0.042)	0.797 (±0.037)
Archipelago	**91**	**11.4 (±0.90)**	**13.40 (±5.49)**	**0.801 (±0.027)**	**0.798 (±0.025)**
FORAGING AGGREGATION					
São Tomé	**23**	11.43 (±1.07)	5.63 (±1.30)	0.778 (±0.040)	0.811 (±0.028)
Príncipe	**7**	7.00 (±0.59)	5.20 (±1.26)	0.788 (±0.044)	0.770 (±0.026)
Archipelago	**30**	**9.21 (±0.74)**	**11.58 (±3.96)**	**0.783 (±0.029)**	**0.790 (±0.019)**

Key: *N* number of samples; *Na* number of alleles; *AR* allelic richness; *H0* observed heterozygosity; *He* expected heterozygosity

Table 2. Summary statistics for $_{mt}$DNA Control Region of São Tomé and Príncipe *Chelonia mydas* rookeries and foraging aggregations (individual islands and archipelago as a whole).

	Population	N	H	Hd (± sd)	Π (± sd)
ROOKERY					
	São Tomé	119	9	0.607 (±0.047)	0.0020 (±0.002)
	Príncipe	19	3	0.433 (±0.117)	0.0001 (±0.009)
	Archipelago	**138**	**9**	**0.590 (±0.044)**	**0.0019 (±0.001)**
FORAGING AGGREGATION					
	São Tomé	36	6	0.602 (±0.052)	0.0020 (±0.002)
	Príncipe	7	3	0.524 (±0.209)	0.0011 (±0.001)
	Archipelago	**43**	**7**	**0.547 (±0.080)**	**0.0019 (±0.003)**

Key: *N* number of samples; *H* number of haplotypes; *Hd* haplotype diversity; *Π* nucleotide diversity; *sd* standard deviation

Population structure and demography

There was no significant genetic differentiation between the rookery of São Tomé and that of Príncipe using either mitochondrial (F_{st} = 0.0017, p > 0.05) or nuclear markers (F_{st} = 0.003, p > 0.05). Based on this overall lack of genetic differentiation between the two islands at both rookery and foraging aggregation levels, we treated each as representing either as a single population, or a single foraging aggregation, respectively (São Tomé and Príncipe, STP) for downstream analyses.

Considering the combined data set, we found that $_{mt}$DNA frequencies at the STP rookery were significantly different from the African rookeries of Ascension and Bioko islands, and Poilão (Guinea-Bissau), as well as all other rookeries included in this study (Table S4, Supporting Information). Estimates of interpopulation migration (number of virtual migrants, Wright, 1978) between STP and other rookeries were low in most cases (Nm < 0.1), being only relevant between STP and the Brazilian rookeries of Atol das Rocas/Noronha (N_m = 4.5), Trindade Island (N_m = 3.54), and the Eastern Atlantic rookeries of Bioko and Ascension (N_m = 2.18 and N_m = 2.30 respectively). In addition, the STP foraging aggregation showed no significant difference in haplotype frequencies with the STP rookery (F_{st} = -0.00895, p = 0.69369), and was found to be significantly differentiated from all foraging aggregations sampled in the Atlantic, with F_{ST} values ranging from 0.032 – 0.841, p < 0.001 (Table S4). Neutrality tests applied to the $_{mt}$DNA dataset revealed non-significant small negative values of Tajima's D (D = -2.60206) and Fu's F_s (F_s = 0.34723) and small positive value of Ramos-Onsins and Rozas' R_2 statistic (R_2 = 0.047), which were in agreement with the relatively stability of *C. mydas* populations of São Tomé and Príncipe inferred by the coalescent BSP analysis (Fig. 3).

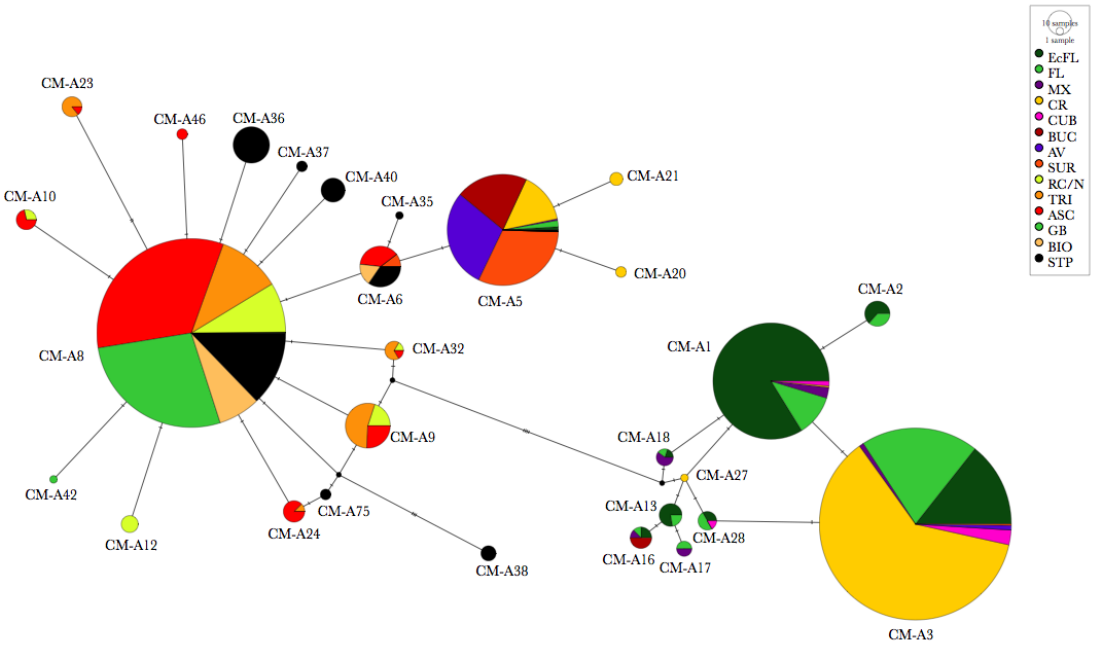

Figure 2. The genealogical relationships between São Tomé and Príncipe's female haplotypes (740 bp) and other *C. mydas* rookeries, as indicated by the median-joining network of mitochondrial control region haplotypes found in the Atlantic. Acronym list and references are included in Tables 1 and 2.

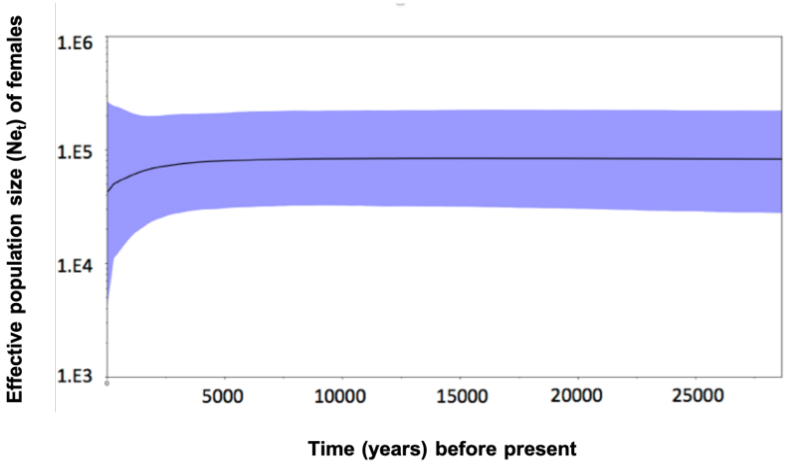

Figure 3. Bayesian skyline plot showing the effective population size fluctuation throughout time of *Chelonia mydas* from São Tomé and Príncipe, West Africa. Solid line represents median estimations; purple area indicates confidence interval.

Mixed Stock Analysis

A strong link between São Tomé and Príncipe's (STP) foraging aggregations and rookery is shown by the MSA results (Fig. 4 and Table S5 – Supporting Information), indicating that São Tomé and Príncipe rookery was the most likely contributor to the local foraging grounds, with an estimated mean contribution of 57%, and maximum contribution up to 72%. However, when we consider the size of the source population as a weighing prior, Guinea Bissau stands out as the highest contributor to STP foraging aggregation, with São Tomé and Príncipe and Ascension islands with similar contributions. In neither case the nearby island of Bioko has any relevant contribution.

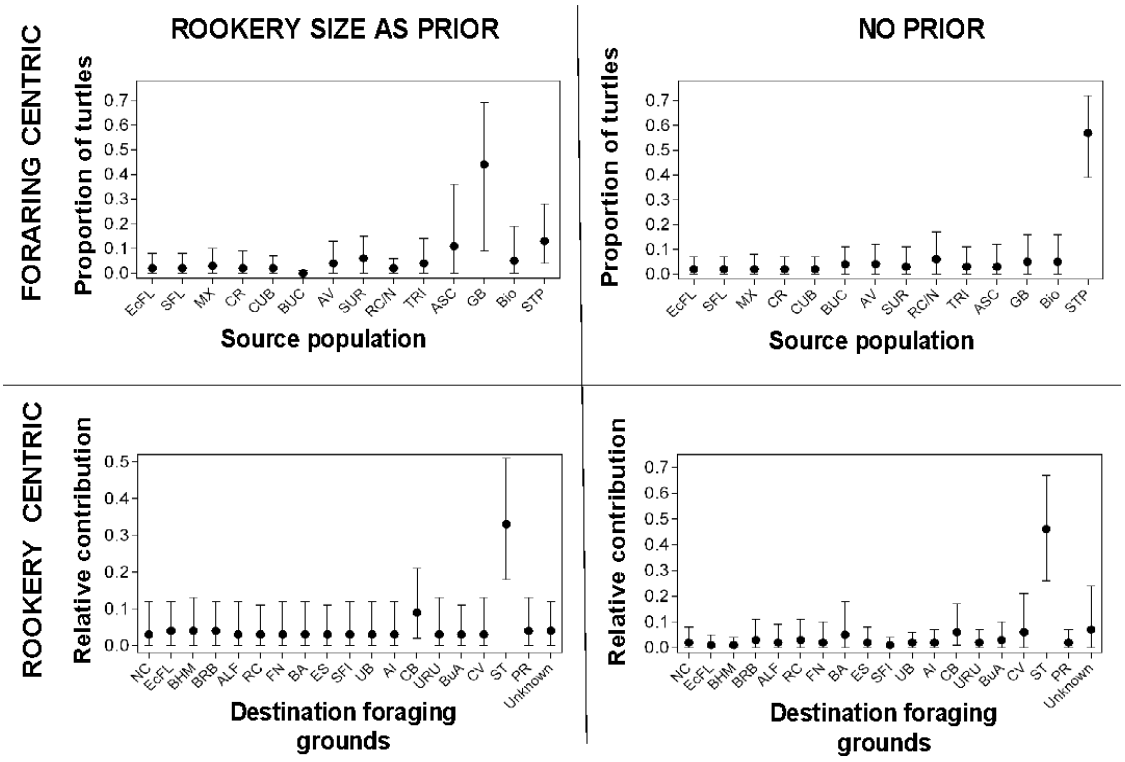

Figure 4. A. Estimated source contributions of Atlantic rookeries to the foraging aggregations of São Tomé and Príncipe. **B**. Estimated contribution of São Tomé and Príncipe rookery to Atlantic foraging grounds. Both figures include estimates *with* and *without* rookery size as a prior. Bars represent 95% confidence intervals. Acronym list and references are included in Tables S1 and S2.

DISCUSSION

Our results provide the most comprehensive analysis to date of genetic diversity of São Tomé and Príncipe's green turtle population. We have expanded previous analyses on green turtle genetic diversity in the region by increasing the number of $_{mt}$DNA sequences for the adult population and adding for the first time nuclear data for both rookery, and foraging aggregations, resulting from the analysis of 11 microsatellite loci. Our results showed that São Tomé and Príncipe islands were not genetically differentiated at either $_{mt}$DNA or nuclear level, suggesting that females nesting on these two islands should be considered as belonging to a single population or rookery. This rookery (when pooling both islands) was significantly differentiated from the others in the Atlantic, including the nearby island of Bioko (approximately 270 Km distant to Príncipe Island), which contradicts the lack of differentiation between these two rookeries reported by Patrício et al. (2017a). The reporting of new haplotypes for São Tomé and Príncipe rookery and the higher sample size used on this study are likely to have contributed to a higher resolution of the levels of diversity for this rookery. Both our nuclear and $_{mt}$DNA data confirm previous findings of Formia et al. (2006), who based solely on $_{mt}$DNA data showed that São Tomé and Príncipe's rookery exhibits high genetic diversity when compared with values reported for other Atlantic rookeries and being especially high when compared to other Eastern Atlantic populations (Tables S2 and S3).

The highest frequency (≈ 60%) of the CM-A8 haplotype in both rookery and foraging aggregations was similar to previous results obtained by Formia et al. (2006) for São Tomé's rookery and also for other Atlantic rookeries and foraging aggregations (Bjorndal et al. 2006; Formia et al. 2007; Naro-Maciel et al. 2006; Proietti et al. 2012). Thus, the proportion of CM-A8 previously detected for this rookery did not vary much with the new sampled individuals, but having a larger sample helps reduce uncertainty around the frequency of the haplotype CM-A36, which was previously only recorded for three individuals by Formia et al. (2006), but at the detected higher frequency in this study (20%) contributes to a more robust rookery contribution estimate to the São Tomé foraging aggregation, and foraging areas in Brazil (Proietti et al. 2009). Additionally, we detected for the first time in a rookery the haplotypes CM-A40 and CM-A75, that were only previously found in other West Africa foraging grounds (CM-A40, A. Formia, *unpublished data*), and in Brazil (CM-A75, Naro-Maciel et al. 2012).

Analysis of historical population demographic changes using neutrality tests and the Bayesian Skyline Plot (BSP) suggested that São Tomé and Príncipe's rookery has been historically stable, with only subtle fluctuations in effective population size. This result is somewhat intriguing in

light of what was previously found by Formia et al. (2006) for the neighbouring island of Bioko, where they found much lower levels of genetic diversity and signals compatible with a unimodal distribution model of a rapidly expanding population. According to these authors, the higher diversity found in São Tomé and Príncipe may have resulted from the impact of a relatively high influx of immigrants and subsequent admixture but also hypothesized that this archipelago corresponds to a remnant of a larger ancestral population in the region. While our results do not allow us to make assumptions about the impact of migratory influx on levels of genetic diversity, the relatively stable demographic history of São Tomé and Príncipe rookery as deduced from $_{mt}$DNA information, suggests a long-term persistence of the environmental conditions of the hydroclimatic zone where these islands are located, that likely functioned as a Pleistocene marine refuge area (Le Loeuff & Von Cosel, 1998).

It is well documented that during the Pleistocene, the cyclical climate changes (glacial and interglacial periods) caused contractions and expansions of the tropical zone in the eastern Atlantic with profound modifications on sea-level and concomitantly on ecological conditions of the region (Le Loeuff & Von Cosel, 1998). These modifications were especially noticeable in Bioko Island, which during the last glaciation period was repeatedly connected to the continent (Rohling et al. 1998). By contrast, the oceanic islands of São Tomé and Príncipe have never been connected to the continent and probably have only suffered very slight alterations on ecological conditions compared to Bioko Island. While based on these available evidences it is possible to deduct that processes of population extinction-recolonization *versus* persistence have shaped the current patterns of genetic variability at Bioko and São Tomé and Príncipe's rookeries, respectively, further studies combining paleoecological and genetic data with species distribution models (SDMs) will be crucial to illuminate this hypothesis.

The juvenile green turtles at the São Tomé and Príncipe's foraging aggregations exhibit high levels of genetic diversity, which are similar to those reported for other aggregates (Bass et al. 2000; Prosdocimi et al. 2012). This result was somewhat expected as these aggregations are typically composed of individuals from mixed stocks. In this study, we found significant differentiation (as revealed by F_{ST}) between São Tomé's foraging aggregation and others sampled in the Atlantic, but similarity between both the rookery and the foraging aggregation. This relative genetic homogeneity found in this foraging aggregation could be explained by the high contribution from the São Tomé and Príncipe's rookeries compared with the negligible contribution from outside rookeries, as evidenced in the foraging-centric Mixed Stock Analysis (MSA) estimates (without rookery size as a prior). The exception to this pattern is the relatively high contribution of the Eastern Atlantic´s largest rookery, Guinea Bissau, when rookery size

was included in the estimates. A previous study performed by Godley et al. (2010), which used sequences from 75 juveniles from São Tomé island found that including only rookeries in the Eastern Atlantic as potential sources, Poilão, in Guinea Bissau, would contribute a maximum of 10% to São Tomé foraging aggregation. This contrasts with our maximum contribution of 40%. However, this last result could be an artifact as the highly frequent and widespread haplotype CM-A8 in eastern Atlantic populations, including São Tomé and Príncipe's foraging aggregation, is (nearly) fixed in the Guinea Bissau rookery (Patrício et al. 2017a), and the inclusion of rookery size as a prior, which to our knowledge, was not used by Godley et al. (2010).

The rookery-centric analysis of our data further reinforces the position of the São Tomé and Príncipe's rookery as the primary source of juveniles foraging in São Tomé, which is consistent with the lack of genetic differentiation between the rookery and the foraging aggregation and the similar levels of genetic diversity found in both juveniles and females. Overall, these results are in agreement with the natal homing behavior and the "closest to home" hypothesis, according to which the immature turtles tend to settle in foraging aggregations closest to their natal home (Bowen and Karl 2007). Despite this general pattern, evidence from our $_{mt}$DNA analysis suggests that connectivity between São Tomé and Príncipe and South Western Atlantic foraging grounds is occurring. For example, the rare haplotype CM-A75 is shared between the foraging ground of Fernando de Noronha (Brazil) and both foraging aggregation and rookery of São Tomé and Príncipe. Moreover, the MSA also suggests that São Tomé and Príncipe rookery contributes, albeit only moderately, as a source population for Cassino Beach (Brazil). These evidences for trans-oceanic connectivity, namely between the Eastern and the South Western Atlantic, are consistent with results from previous studies based on mark-recapture and telemetry analysis (Pritchard, 1973; Luschi et al. 1998; Marcovaldi et al. 2000; Grossman et al. 2007), as well as other MSA studies (Naro-Maciel et al. 2006; Proietti et al. 2009; Monzón-Argüello et al. 2010; Patrício et al. 2017a). Moreover, according to a study by Scott et al. (2017), dynamic oceanic conditions in the Gulf of Guinea result in seasonal dispersion variability driven by wind changes arising from the yearly north/southward migration of the intertropical convergence zone. This results in varying degrees of hatchling retention, with increasing westerly dispersion of hatchlings throughout the hatching season, with the majority of simulated hatchlings dispersing west into the South Atlantic Ocean with the South Equatorial Current. This pattern of dispersal in the Gulf of Guinea is evident by the higher migration rates between São Tomé and Príncipe rookery and Brazil than with the Eastern Atlantic rookeries, assuming that the estimated number of migrants (Nm) greater than 1–4 indicate that gene flow is sufficient to maintain a relatively homogeneous gene pool (Slatkin, 1987).

CONCLUSIONS AND CONSERVATION IMPLICATIONS

Understanding patterns of connectivity and dispersal among species with complex life cycles and exposure to multiple threats such as marine turtles, is crucial for prioritizing conservation and management measures. Assessing the genetic diversity of green turtles from the São Tomé and Príncipe archipelago through a combination of genetic markers, we showed that nesting and foraging turtles found on these islands exhibit relatively high levels of genetic diversity, representing an important genetic pool in the region. Moreover, the high genetic differentiation found between this archipelago's turtle population (both foraging and nesting) and others from the Atlantic suggests that this archipelago should be defined in the future an important conservation management unit. Although the use of a different type of genetic marker provided additional insight into our knowledge about the genetic structure of green turtle rookery and foraging aggregations in São Tomé and Príncipe, assessing small-scale patterns of connectivity, at the regional level (e.g. Gulf of Guinea), and between ocean basis would benefit greatly by an increased effort in sampling other East African rookeries, and the use of nuclear markers, such as microsatellites. Although important gaps persist in our knowledge about sea turtle ecology, it is well documented that ocean currents strongly affect the migratory behavior and dispersal pathways of this organisms (Luschi et al. 2003; Mansfield et al. 2017). While it is likely that the strong relationship found here between the rookery and foraging aggregation is linked to the effects of major oceanic currents, namely the Gulf and the South Equatorial currents (Luschi et al. 1998; Scott et al. 2017), further analyses based on satellite technologies and novel numerical simulation models (e.g. Briscoe et al. 2018) could be crucial to test whether the "closest to home" hypothesis (Bolker et al. 2007) fits the population dynamics of São Tomé and Príncipe. Improving our knowledge on patterns of connectivity and demography of the species in this region, will ultimately lead to an integrative and effective conservation and management plan.

Acknowledgements

Fanio, João, Nicho, Y. Yanes-Perez, A. Santos, A. Besugo & M. Branco (São Tomé); H. Carvalho, J. Pereira and I. Prazeres (Príncipe); S. Lopes, P. Ribeiro, S. Silva and A. Bessa (CTM/CIBIO). Permits were provided by National Agency for the Environment (DGA) and Eng. Arlindo de Carvalho. This study was funded by national funds through FCT - Fundação para a Ciência e a Tecnologia by a doctoral scholarship awarded to JMH (PD/BD/52599/2014). HG is supported by a postdoctoral Grant from FCT (SFRH/BPD/102966/2014), and FS is supported by FEDER (COMPETE, POCI-01-0145-FEDER-006821) and FCT (UID/BIA/50027/2013) funds. All samples used in this study were collected and exported under the CITES permits 15ST00009/AC; 17ST000001/AC.

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SUPPORTING INFORMATION

Table S1. Identification of 14 microsatellite loci, with indication of allele size range, fluorescent labelling, and amplification multiplex panel. More details in Methods.

Locus	Reference	Size range (bp)	Flurophore[a]	Multiplex panel
CcP7D04	Shamblin et al. 2009	>500	6-FAM	1
ERIM25	Shamblin et al. 2013	215-255	VIC	1
CCP7C06*	Shamblin et al. 2009	339-375	VIC	1
Cc5H07	Shamblin et al. 2009	220-276	NED	1
Cc1F01	Shamblin et al. 2009	304-340	NED	1
CcP2F11	Shamblin et al. 2009	266-310	PET	1
Cc1G02	Shamblin et al. 2009	258-334	6-FAM	1
Eim09	Miro-Herrans et al. 2008	277-294	6-FAM	2
Eim40	Miro-Herrans et al. 2008	240-248	NED	2
ERIM03	Shamblin et al. 2013	210-220	6-FAM	2
ERIM22*	Shamblin et al. 2013	374-422	6-FAM	2
ERIM21	Shamblin et al. 2013	208-220	VIC	2
ERIM19*	Shamblin et al. 2013	266-274	PET	2
Cc5C08	Shamblin et al. 2009	289-379	VIC	2

[a] Forward primers were modified at the 5´end with a fluorescent label: 6-FAM (blue, NED (yellow), VIC (green) or PET (red);

*Microsatellite loci not used in analysis due to the potential existence of null alleles, or large allele dropout.

Table S2. Levels of genetic diversity found in Chelonia mydas in São Tomé and Príncipe islands (rookery and foraging grounds) based on 11microsatellite loci (this study), and in other Atlantic populations.

Population	Acronym	N	Na (± se)	AR (± se)	Ho (± se)	He (± se)	Reference
ROOKERIES							
São Tomé & Príncipe	**STP**	**91**	**11.4 (±0.90)**	**13.40 (±5.49)**	**0.801 (±0.027)**	**0.798 (±0.025)**	**This study**
Florida	FL	49	13.7 (±8.25)	11.93 (±6.40)	0.792 (±0.015)	0.808 (±0.029)	Seminoff (2004)
Tortuguero	CR	58	13.9 (±7.42)	12.01 (±5.84)	0.818 (±0.013)	0.815 (±0.027)	Seminoff (2004)
Surinam	SUR	49	13.3 (±6.07)	11.91 (±4.89)	0.835 (±0.014)	0.822 (±0.024)	Seminoff (2004)
Aves	AV	33	10.5 (±4.69)	10.17 (±4.50)	0.788 (±0.018)	0.767 (±0.036)	Penaloza (2000)
Rocas Atoll	RC	30	11.1 (±4.50)	10.98 (±4.46)	0.804 (±0.019)	0.783 (±0.031)	Bellini et al. (1995)
Trindade Island	TRI	82	13.5 (±6.64)	10.82 (±4.85)	0.787 (±0.012)	0.780 (±0.036)	Almeida et al. (2011)
Ascension Island	ASC	46	17.8 (±nd)	nd	0.668	nd	Roberts et al. (2004)
Atol das Rocas	RC	41	14.8 (±nd)	nd	0.702	nd	Roberts et al. (2004)
Aves Island	AV	44	17.5 (±nd)	nd	0.592	nd	Roberts et al. (2004)
Cyprus	CYP	25	11 (±nd)	nd	0.660	nd	Roberts et al. (2004)
Mexico	MX	7	7.5 (±nd)	nd	0.857	nd	Roberts et al. (2004)
FORAGING AGGREGATION							
São Tomé and Príncipe	**STP**	30	**9.21 (±0.74)**	**11.58 (±3.96)**	**0.783 (±0.029)**	**0.790 (±0.019)**	**This study**

Key: *N* number of samples; *Na* mean Number of Alleles; *AR* allelic richness; *Ho* observed heterozygosity; *He* expected heterozygosity; *se* standard error; *nd* no data

Table S3. Summary statistics for mtDNA Control Region of Chelonia mydas Atlantic rookeries and foraging grounds used in this study. São Tomé and Príncipe islands are depicted individually (ST and PCP) and as a single rookery or foraging aggregation (STP).

Location	ACR	RMU*	nS	H	Hd (± sd)	Π (± sd)	References
ROOKERIES							
East Central Florida	EcFL	NW	311	9	0.512 (±0.020)	0.0016 (±0.001)	Shamblin et al. 2015
South Florida	SFL	NW	174	10	0.444 (±0.043)	0.0022 (±0.002)	Shamblin et al. 2015
Mexico	MEX	NW	20	7	0.816 (±0.058)	0.0051 (±0.003)	Encalada et al. 1996
Cuba	CUB	NW	28	7	0.648 (±0.089)	0.0053 (±0.003)	Ruiz-Urquiola et al. 2010
Costa Rica	CR	NW	433	5	0.163 (±0.023)	0.0033 (±0.002)	Encalada et al. 1996, Bjorndal et al. 2006
Suriname	SUR	C	73	4	0.132 (±0.053)	0.0013 (±0.001)	Encalada et al. 1996, Shamblin et al. 2012
Buck Island	BUC	C	49	2	0.153 (±0.065)	0.0030 (±0.002)	Shamblin et al. 2012
Aves Island	AV	C	67	2	0.140 (±0.055)	0.0029 (±0.002)	Lahanas et al. 1994, 1998, Shamblin et al. 2012
Atol das Rocas/ Fernando de Noronha	RC/N	SW/SC	69	7	0.463 (±0.071)	0.0026 (±0.002)	Encalada et al. 1996, Bjorndal et al. 2006
Trindade	TRI	SW/SC	99	7	0.505 (±0.051)	0.0012 (±0.001)	Bjorndal et al. 2006
Ascension Is.	ASC	SC	245	13	0.303 (±0.038)	0.0008 (±0.001)	Encalada et al. 1996, Formia et al. 2007
Guinea Bissau	POI	E/SC	171	2	0.012 (±0.011)	0.0001 (±0.001)	Patricio et al. 2017a
Bioko Island	BIO	E/SC	50	2	0.184 (±0.068)	0.0004 (±0.001)	Formia et al. 2006
São Tomé	**ST**	**E/SC**	**119**	**9**	**0.607 (±0.047)**	**0.0020 (±0.002)**	**Formia et al. 2006; this study**
Príncipe	**PCP**	**E/SC**	**19**	**3**	**0.433 (±0.117)**	**0.0001 (±0.009)**	**This study**
São Tomé and Príncipe	**STP**	**E/SC**	**138**	**9**	**0.590 (±0.044)**	**0.0019 (±0.001)**	**Formia et al. 2006; this study**

Table S3. (Cont.)

FORAGING AGGREGATIONS							
North Carolina	NC	NW	106	12	0.729 (±0.030)	0.0050 (±0.003)	Bass et al. 2006
East C. Florida	EcFL	NW	62	6	0.486 (±0.067)	0.0031 (±0.002)	Bass et al. 2006
Bahamas	BHM	NW	80	7	0.612 (±0.018)	0.0060 (±0.003)	Lahanas et al. 1998
Puerto Rico	PR	NW	103	10	0.680 (±0.040)	0.0080 (±0.005)	Patrício et al. 2017b
Barbados	BRB	SW	60	8	0.773 (±0.028)	0.0105 (±0.006)	Luke et al. 2003
Almofala	ALF	SW	117	13	0.717 (±0.031)	0.0067 (±0.004)	Naro-Maciel et al. 2007
Atol Rocas	RC	SW	101	8	-	-	Bjorndal et al. 2006
F. Noronha	FN	SW	117	12	0.650 (±0.028)	0.0040 (±0.003)	Naro-Manciel et al. 2012
Cassino Beach	RC	SW	101	12	0.586 (±0.050)	0.0020 (±0.002)	Proietti et al. 2012
Arvoredo Is	AI	SW	115	12	0.583 (±0.045)	0.0024 (±0.001)	Proietti et al. 2009
Ubatuba	UB	SW	113	10	0.446 (±0.056)	0.0021 (±0.002)	Naro-Maciel et al. 2007
Bahia	BA	SW	45	6	0.648 (±0.053)	0.0020 (±0.002)	Naro-Manciel et al. 2012
Espírito S.	ES	SW	157	9	0.595 (±0.031)	0.0030 (±0.002)	Naro-Manciel et al. 2012
S. F. Itabapoana	SFI	SW	190	13	0.493 (± 0.038)	0.0014 (±0.001)	Costa Jordão et al. 2017
Buenos Aires	BuA	SW	93	9	0.553 (±0.051)	0.0020 (±0.002)	Prosdocimi et al. 2012
Uruguay	UGY	SW	144	10	0.387 (±0.051)	0.0014 (±0.001)	Caraccio, 2008
Cape Verde	CV	E	44	5	0.558 (±0.045)	0.0040 (±0.003)	Monzón-Arguello et al. 2010
São Tomé	**ST**	**E/SC**	**36**	**6**	**0.602 (±0.052)**	**0.0020 (±0.002)**	**This study**
Príncipe	**PCP**	**E/SC**	**7**	**3**	**0.524 (±0.209)**	**0.0011 (±0.001)**	**This study**
São Tomé and Príncipe	**STP**	**E/SC**	**43**	**7**	**0.547 (±0.080)**	**0.0019 (±0.003)**	**This study**

Table S4. Pairwise comparisons of *Chelonia mydas* populations in the Atlantic: A - Rookeries; B - Foraging Grounds. FST values are shown below the diagonal, and p-values for exact tests of differentiation above the diagonal. Non-significant values ($p > 0.05$) in **bold**. Negative values in *italic*. Acronym list are included in Table 1.

A. ROOKERIES

	STP	RC/N	BIO	ASC	GB	TRI	SUR	AV	BUC	MX	EcFL	SFL	CR	CUB
STP	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RC/N	0.054	-	0.000	0.018	0.000	**0.135**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
BIO	0.103	0.064	-	**0.171**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ASC	0.098	0.028	0.006	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GB	0.255	0.239	0.149	0.073	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TRI	0.066	0.011	0.102	0.066	0.267	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SUR	0.573	0.706	0.846	0.747	0.951	0.663	-	**0.225**	**0.144**	0.000	0.000	0.000	0.000	0.000
AV	0.566	0.697	0.841	0.743	0.951	0.654	0.006	-	**0.063**	0.000	0.000	0.000	0.000	0.000
BUC	0.546	0.674	0.832	0.735	0.956	0.632	0.014	0.024	-	0.000	0.000	0.000	0.000	0.000
MX	0.312	0.405	0.586	0.571	0.867	0.384	0.640	0.617	0.582	-	0.000	0.000	0.000	0.000
EcFL	0.445	0.504	0.581	0.584	0.687	0.490	0.602	0.591	0.587	0.081	-	0.000	0.000	0.000
SFL	0.475	0.549	0.639	0.634	0.771	0.530	0.659	0.637	0.640	0.223	0.298	-	0.000	0.000
CR	0.689	0.772	0.834	0.782	0.884	0.747	0.830	0.817	0.827	0.608	0.560	0.109	-	0.000
CUB	0.374	0.464	0.628	0.611	0.871	0.443	0.690	0.664	0.646	0.110	0.262	0.046	0.299	-

B. FORAGING GROUNDS

	STP	RC	FN	ALF	BA	ES	UB	AI	CB	SFI	UGY	BUA	CV	NC	ECFL	BHM	BRB	PR
STP	-	0.000	0.000	0.009	0.009	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RC	0.078	-	0.018	**0.216**	**0.072**	0.027	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000
FN	0.151	0.030	-	0.000	**0.117**	0.018	0.000	0.009	0.000	0.000	0.000	0.000	**0.703**	0.000	0.000	0.000	0.000	0.000
ALF	0.093	0.005	0.054	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000
BA	0.121	0.022	0.015	0.060	-	**0.901**	0.000	**0.261**	**0.099**	**0.054**	0.000	**0.171**	0.000	0.000	0.000	0.000	0.000	0.000
ES	0.106	0.026	0.024	0.073	*0.011*	-	0.000	**0.135**	0.018	0.027	0.000	**0.108**	0.018	0.000	0.000	0.000	0.000	0.000
UB	0.032	0.044	0.119	0.075	0.065	0.054	-	**0.090**	**0.153**	**0.126**	**0.640**	0.036	0.000	0.000	0.000	0.000	0.000	0.000
AI	0.056	0.028	0.060	0.069	0.005	0.007	0.011	-	**0.946**	**0.901**	0.009	**0.982**	0.000	0.000	0.000	0.000	0.000	0.000
CB	0.040	0.037	0.073	0.075	0.015	0.017	0.007	*0.006*	-	**0.937**	0.0180	**0.838**	0.000	0.000	0.000	0.000	0.000	0.000
SFI	0.051	0.039	0.081	0.084	0.019	0.018	0.006	*0.005*	0.005	-	0.009	**0.892**	0.000	0.000	0.000	0.000	0.000	0.000
UGY	0.038	0.079	0.167	0.110	0.114	0.088	*0.003*	0.031	0.021	0.021	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000
BUA	0.061	0.031	0.062	0.071	0.006	0.008	0.013	*0.008*	0.007	*0.006*	0.034	-	0.000	0.000	0.000	0.000	0.000	0.000
CV	0.231	0.048	*0.009*	0.060	0.047	0.060	0.200	0.116	0.131	0.147	0.281	0.120	-	0.000	0.000	0.000	0.000	0.000
NC	0.724	0.643	0.689	0.552	0.721	0.754	0.749	0.744	0.741	0.769	0.784	0.744	0.682	-	0.018	0.000	0.000	0.000
ECFL	0.841	0.731	0.768	0.632	0.833	0.834	0.846	0.834	0.833	0.846	0.877	0.841	0.785	0.035	-	0.027	0.000	0.000
BHM	0.692	0.605	0.654	0.508	0.684	0.731	0.728	0.720	0.716	0.751	0.770	0.719	0.632	0.051	0.050	-	0.000	**0.181**
BRB	0.360	0.258	0.333	0.165	0.346	0.431	0.413	0.409	0.404	0.462	0.479	0.403	0.290	0.227	0.324	0.179	-	**0.091**
PR	0.563	0.484	0.541	0.393	0.555	0.622	0.607	0.603	0.598	0.646	0.654	0.598	0.505	0.046	0.101	0.022	0.773	-

Table S5. Rookery contribution estimates to juvenile green turtles foraging aggregations in São Tomé and Príncipe waters, based on mixed stock analysis weighted by rookery size (A) and no weighing prior (B). Values of 0.00 represent no contribution. Rookery source acronyms are explained in Table 2.

	A. With rookery size as prior			B. No prior		
	Mean	**97.5%**	**2.5%**	**Mean**	**97.5%**	**2.5%**
EcFL	0.02	0.08	0.00	0.02	0.07	0.00
SFL	0.02	0.08	0.00	0.02	0.07	0.00
MX	0.03	0.10	0.00	0.02	0.08	0.00
CR	0.02	0.09	0.00	0.02	0.07	0.00
CUB	0.02	0.07	0.00	0.02	0.07	0.00
BUC	0.00	0.01	0.00	0.04	0.11	0.00
AV	0.04	0.13	0.00	0.04	0.12	0.00
SUR	0.06	0.15	0.00	0.03	0.11	0.00
RC/N	0.02	0.06	0.00	0.06	0.17	0.00
TRI	0.04	0.14	0.00	0.03	0.11	0.00
ASC	0.11	0.36	0.00	0.03	0.12	0.00
GB	0.44	0.69	0.09	0.05	0.16	0.00
Bio	0.05	0.19	0.00	0.05	0.16	0.00
STP	0.13	0.28	0.04	0.57	0.72	0.39

PAPER 2

Stable isotopes reveal dietary differences and site fidelity in juvenile green turtles foraging around São Tomé island, West Central Africa

J.M. Hancock, S. Vieira, V. Jimenez, J. Carvalho Rio and R. Rebelo

Lúcio e família - Praia Gamboa
(São Tomé)

Inocêncio dos Prazeres (Nuno Kuto) –
Nova Estrela
(Príncipe)

Aladino Santos (Didy) – Porto Alegre
São Tomé

"And the turtles, of course all the turtles are free, as turtles and, maybe, all creatures should be".

Dr. Seuss

Hancock, J. M., Vieira, S., Jimenez, V., Rio, J. C., & Rebelo, R. (2018). Stable isotopes reveal dietary differences and site fidelity in juvenile green turtles foraging around São Tomé Island, West Central Africa. *Marine Ecology Progress Series*, *600*, 165-177.

Stable isotopes reveal dietary differences and site fidelity in juvenile green turtles foraging around São Tomé island, West Central Africa

ABSTRACT

Green sea turtles are common in West Central Africa, but little is known about the occurrence of immatures in foraging grounds in the Gulf of Guinea islands, known for their volcanic origin and narrow coastal fringes. This study presents results of in-water surveys foraging grounds off São Tomé island, in São Tomé and Príncipe archipelago, providing the first available data on the size distribution of immature green sea turtles of different life-stage groups on these islands. Two sites offering distinct types of food sources were studied, and isotopic signatures of immature turtles hand-captured at each foraging site were used to infer establishment duration at the foraging sites and diet preferences. Size at recruitment in the region was estimated to occur at a minimum size of 34 cm CCL, and resident immature turtles ranged from 53 to 87 cm CCL. Immatures sampled at each site showed clear differences in isotopic signatures, suggesting that they establish specific home ranges related to the available diet items and use them for extended periods of at least several months. Macroalgae were as or more important than seagrasses for the turtle's diets, and there was evidence that these individuals are not strictly herbivorous. Our study provides the first data set to which to compare demographic data from other locations in West Africa, where current knowledge on green turtle foraging behavior is limited or non-existent and indicates that even oceanic islands that are geologically recent like São Tomé may provide important recruitment/development habitats for juvenile green turtles.

Keywords: *Chelonia mydas*, Settlement, Stable Isotopes, Foraging Ecology

INTRODUCTION

Studies on sea turtle biology have typically focused on the reproduction and post-nesting movements of females, logistically more accessible. Only more recently multi-disciplinary approaches have tackled the lives of cryptic life stages such as males and immatures and their use of neritic foraging habitats (Rees et al. 2016). population assessments at the foraging grounds providing local size-class distributions may contribute information essential for establishing population abundance trends (Seminoff et al. 2003; Bjorndal et al. 2005; Bjorndal et al. 2010). Furthermore, knowledge of resource use can help determine the importance of different marine habitats for the different turtle life stages and improve our understanding of migratory connectivity among breeding, foraging, and developmental habitats (e.g. Bradshaw et al. 2017).

The analysis of stable isotopes of both sea turtles and their diets has been increasingly combined with in-water population assessments to study foraging behaviour and resource use due to their high versatility. Stable isotope ratios in the tissues of consumers reflect those of their diet in a predictable manner (Hobson, 1999; 2007; Post, 2002); the ratio of nitrogen isotopes ($\delta\,^{15}N$) increases along each trophic transfer and can be used to estimate trophic position of organisms (Minagawa & Wada, 1984; Peterson & Fry, 1987; Post, 2002b), while the ratio of carbon isotopes ($\delta\,^{13}C$) varies substantially among primary producers with different photosynthetic pathways, and thus can be used to determine the sources of dietary carbon (deNiro & Epstein, 1978). Additionally, as both Carbon and Nitrogen stable isotope ratios at the base of food webs vary spatially, this is reflected in spatial variability in isotopic composition among food webs (Jennings et al. 1997; Finlay 2001; Bearhop et al. 2004; Graham et al. 2010). The quantification of stable isotopes is thus particularly useful in studying ontogenic shifts in sea turtle foraging strategies (Arthur et al. 2008; Shimada et al. 2014; Ramirez et al. 2015; Velez-Rubio et al. 2016; Tomaszewicz et al. 2017), identifying the geographic location of foraging habitats (Dodge et al. 2011; Ceriani et al. 2014; López-Castro et al. 2013, 2014), as well as clarifying sea turtle trophic position and resource use (Lemons et al. 2011; Hall et al. 2015; Pajuelo et al. 2016; Sampson et al. 2017).

In West Central Africa, two green turtle regional management units overlap (South Central and Eastern Atlantic, Wallace et al. 2010) where turtles are exposed to multiple threats both on nesting and foraging grounds (Formia et al. 2003; Carranza et al. 2006; Fitzgerald et al. 2011; Riskas & Tiwari, 2013). Foraging grounds, mostly used by immature green sea turtles, have been identified in the continental countries, specifically in Cameroon, Republic of Congo and

Gabon (Formia, 2002; Formia et al. 2003, 2006; Hyacinthe et al. 2012; A. Girard, *pers. comm.*), but not on the islands of the Gulf of Guinea. These islands, including Bioko and Annobón (Equatorial Guinea) and São Tomé and Príncipe, are of volcanic origin dating 15,7 million years ago (Deruelle et al. 1991), and display high relief, resulting in very narrow littoral fringes (Juste & Fa, 1994). We conducted this study aiming to provide the first accounts on spatial and temporal aspects of local aggregations of immature green sea turtles foraging on the Gulf of Guinea islands using in-water surveys, and to assess possible patterns of resource use using stable isotope analysis. We sampled individuals at two distinct habitats (seagrass vs. macroalgae) on São Tomé island and investigated how the use of these habitats by different size classes could be reflected on their isotopic niches. We sought validation of our results by (1) comparing the isotopic signatures of the immature, presumably local, individuals with those of breeding females, as female signatures should represent distant foraging grounds visited in the months preceding their migration (Stearns, 2002) and (2) sampling a selection of putative diet items to obtain clues about preferred diets and resource use by potentially resident immatures. This dataset offers an insight into green turtle recruitment and settlement dynamics in the Gulf of Guinea islands and will be the first data set to which to compare demographic data from other locations in West Africa, where current knowledge on green turtle foraging behaviour is limited or non-existent.

METHODS

Study Sites

São Tomé Island is one of the two islands comprising the small, insular country of São Tomé and Príncipe that is located in the Gulf of Guinea, West Africa, approximately 250 km off the continental mainland. The littoral fringe surrounding the island covers approximately 450 km^2 above the 200 m isobar (Afonso et al. 1999).

Informal interviews were conducted with spear fishermen, turtle hunters and fish sellers in the main coastal communities of the island throughout 2014 and 2015, with the aim of identifying known sea turtle aggregation areas or historical hunting grounds, as well as potential diet items that may be primarily consumed by the turtles using those areas. An island-wide survey of sites presumed to offer either suitable foraging habitat (including the existence of extensive, shallow macroalgae or seagrass banks), availability of shelter and/or resting areas and evidence of the all-year-round presence of sea turtles was conducted by boat over two days in September 2015.

The survey covered the entire coastline and was carried out with the participation of local spear fishermen and turtle hunters. All sites that were visited and visually inspected by snorkeling are depicted on Fig.1; we considered as "potential foraging/aggregation sites" those where we could not confirm the presence of turtles. Two areas where sea turtles were observed feeding or resting were selected for this study, and included Ilhéu das Cabras site (Northern Foraging Ground, FGN, 0°21.802'N, 6°45.402'E); and Porto Alegre (Southern Foraging Ground, FGS), with two sub-sites: Praia Cabana, (FGS_{CAB}, 0°1.310'N, 6°31.407'E) and Ponta Santo António (FGS_{PSA}, 0°0.408'N, 6°31.622'E) (Fig.1).

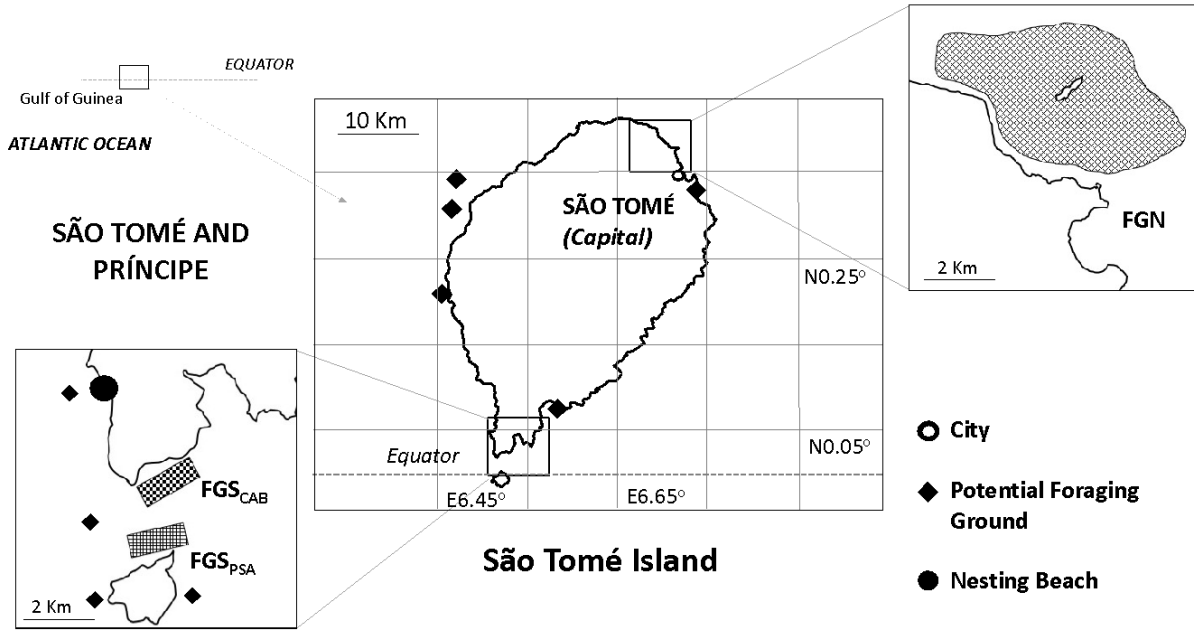

Figure 1. Location of the two foraging grounds (FG) and the nesting beach (Jalé) sampled during this study, as well as potential foraging grounds (sites identified by local fishermen for which no data is available) during two-large scale surveys conducted in 2015 (Area and location of FGN obtained from Alexandre et al. 2017).

For each site the predominant habitat type and associated algae or seagrass species was assessed visually. Average depth was calculated taking several readings using a depth gauge and approximate area was estimated using QGis; a short description of the sites is provided on Table 1.

Table 1. Location and habitat characterization of the study sites

Site	Sub-Site	Location	Habitat type	Distance to shore / Area Surveyed	Range of Depths	Dominant plant/ algal species
FGN (North)	Ilhéu das Cabras	0°21.802'N 6°45.402'E	Seagrass patches	2000 m 1500 ha*	4 – 7 m	Seagrass (*Halodule wrightii*) Macroalgae (*Dyctiota* spp; *Caulerpa* spp)
FGS (South)	Praia Cabana (FGS_{CAB})	0°1.310'N, 6°31.407'E	Rocky reef	200 m 55 ha	6 – 10 m	Macroalgae (*Dyctiota* spp)
	Ponta S. António (FGS_{PSA})	0°0.408'N, 6°31.622'E	Rocky platform	500 m 40 ha	8 – 15 m	Macroalgae (*Polysiphonia* spp, *Dyctiota* spp)

** Alexandre et al. (2017)*

In-Water Visual Surveys

Efforts to document sea turtle presence were carried out between November and February 2016 and 2017 and included (a) in-water visual daytime surveys either by snorkeling (underwater) or at the surface (from a boat) and (b) hand capture of live turtles during daytime (opportunistic) and night (targeted) surveys. The survey methods were adapted to the characteristics of each site, such as habitat type, depth, area and water visibility (e.g. Roos et al. 2005; Mancini et al. 2015). The southern foraging or aggregation areas (FGS_{CAB} and FGS_{PSA}) were associated with rocky areas of spurs and groves at 8-12 m depth that offered resting and hiding areas for turtles, and dense macroalgae mats. Here we conducted underwater visual surveys (10 and 5 transects performed at FGS_{CAB} and FGS_{PSA} respectively), consisting of belt transects following Roos et al. (2005). On these transects, two surface swimmers moved parallel to each other at the same speed, along one contiguous strip approximately 30 m wide (determined by the underwater visibility) and approximately 500 m long, parallel to the shore, resulting in approximately 3 ha covered in each survey. Each transect was usually covered within thirty minutes, depending on surface currents. In the shallow seagrass dominated site at FGN (< 7 m depth) where turtles can be easily seen from the boat, two surveys were conducted from the boat only under conditions

of excellent water visibility, following an expanding square search pattern to maximize the area covered (e.g. Bell et al. 1990; Christman et al. 2013; Acebes et al. 2016), covering an area of approximately 200 ha during each survey, and lasting approximately 60 minutes each.

At all sites, every time a turtle was sighted, the turtle's behaviour (swimming, resting or feeding) and approximate size class was observed and noted, and the location was recorded using a hand-held GPS. When possible, males were identified by their external sexual characteristics (Wyneken & Witherington, 2001). Sighting data was used to calculate capture per unit effort (CPUE) and to assess habitat use; size-classes present at each site were only evaluated after hand-capture of individual turtles (see "Sea Turtle Capture and Handling").

Sea Turtle Capture and Handling

Immature and adult female turtles were sampled for this study at two foraging sites and one nesting beach respectively**.** Each turtle sampled had the minimum curved carapace length (CCL_{min}; notch to notch, ± 0.1 cm) measured using a flexible measuring tape, and was double tagged with Inconel tags (Style 681; National Band and Tag Company, Newport, Kentucky); one tag in the second large proximal scale of each front flipper. Tissue samples were collected from the trailing edge of the rear flipper of each turtle using a sterile razor scalpel and stored in 96% ethanol until processing in the lab. All seized turtles were released on-site within 30 minutes of capture. Turtle sampling methods are as described below:

Immatures All immatures were hand-captured; due to the distinct characteristics of each site, we employed different approaches to capture turtles: at FGN we used the rodeo technique (Ehrhart & Ogren, 1999), in which one person jumped into the water and attempted to capture the turtles as they were sighted at or near the surface or resting at the bottom of the sea; at FGS we selected Cabana (FGS_{CAB}) for targeted hand captures by free-diving after dusk, as turtles were easily found resting under rocky ledges, or well camouflaged amongst the macroalgae beds at this time of the day (JM Hancock, *pers. obs.*). Hand captures at both sites were always performed by holding the anterior and posterior medial section of the turtle's carapace, pulling it out of the water by a slow, vertical ascension, lifting its head to keep the front flippers out of the water until it could be safely hoisted onto the boat, a method that has been shown to be safe for juvenile turtles in several previous studies (e.g., van Dam & Diez, 1998; Ehrhart & Ogren, 1999).

Adult Females Adult female turtles were sampled at Jalé beach (0°2.496'N, 6°30.734'E), the main nesting site for this species in the island of São Tomé, during night patrols conducted by the technical staff of the project "Programa Tatô" of São Tomé during the same period.

Sampling of putative diet items

To obtain the reference isotope ratios for different trophic levels of the foraging grounds' communities and investigate the variation of isotopic ratios at a local scale, we collected samples of the main algae and plant species that were referred by turtle fishermen as being either commonly consumed by green sea turtles or that were most abundant in each sampling location (see Table 1). Because incorporating too many sources would reduce the resolution of mixing models, and we were interested in assessing the differential contribution of plant/algal and animal diets for different sea turtle size groups, we selected the most common plants/algae at each site, as well as the most common primary consumer/ omnivore invertebrate. These included four species of macroalgae of different groups (*Caulerpa sp.* among Chlorophyta*, Dictyota sp.* and *Sargassum sp.* among Phaeophyceae, and *Polysiphonia sp.* among Rhodophyta), one species of seagrass (*Halodule wrightii*) and the common intertidal crab *Grapsus adscensionis* (Osbeck, 1765). The macroalgae *Dictyota sp*. and the crab were the only putative diet items common at both foraging grounds, and so were collected near FGS_{CAB} (Inhame beach, 0°1.464'N, 6°31.147'E) and FGN (Gamboa beach, 0°22.789'N, 6°43.173'E) to identify a possible North-South isotopic distinctiveness in $\delta^{13}C$ values. Crab samples were stored in 96% ethanol until processing. Macroalgae and seagrass samples were stored in a hypersaline solution (2:1 saltwater/salt, as suggested by Tsuda et al. 1985) instead of ethanol since algal material will lose pigments and become very brittle quickly if stored in ethanol, no other fixative was available, and freezing was not possible. Preserved algae samples were kept in the dark and refrigerated (± 4 ºC) until processing.

Stable isotope analysis

From each sea turtle sample, 0.10 – 0.25 g of the epidermis (i.e., *stratum corneum*) was carefully separated from any connective tissue, rinsed with deionized water, finely diced with a scalpel blade, weighed and oven dried for at least 12 h at 60 ºC. Samples of putative diet items were carefully rinsed with de-ionized water until all salt was removed, scraped gently to remove any debris or epiphytes and finely shredded with a scalpel blade, and oven dried as described above. The isotopic signature of the putative diet items was determined using 3 - 5 replicate samples from each item. Lipid extraction was performed on all samples, using a solvent mixture of chloroform/methanol (2 : 1) to a final volume 3 - 5 times the volume of the tissue sample

(approximately 1 g of tissue in 5 ml of solvent mixture). Samples were first centrifuged in an Eppendorf Centrifuge model 5403 for 1 minute at 3400 rpm and left to rest for 30 minutes. After a second centrifugation for 10 minutes at 4 ºC and at the same speed, the supernatant was entirely removed. The previous step was repeated at least three times until the supernatant was clear, then the remaining sample was oven dried for at least 12 h at 60 ºC to remove any residual solvent. Sub-samples of prepared tissue (0.75 – 1.0 mg of animal material, 4 – 5 mg of plant material) were weighed with a microbalance and packed in tin capsules for mass spectrometric analysis.

The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios ($\delta^{13}C$ and $\delta^{15}N$ respectively) in the samples were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston & Owens, 1983), on a Sercon Hydra 20 - 22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyzer for online sample preparation by Dumas-combustion. The standards used were Protein Standard OAS, Sorghum Flour Standard OAS (Elemental Microanalysis, UK) and IAEA-N1 (IAEA, Vienna, Austria) for nitrogen and carbon isotope ratio; $\delta^{15}N$ results were referred to Air and $\delta^{13}C$ to PeeDee Belemnite (PDB). The precision of the isotope ratio analysis, calculated using values from 6 to 9 replicates of standard laboratory material interspersed among samples in every batch analysis, was ≤ 0.2 ‰.

Analytical methodology

We calculated catch per unit effort (CPUE) at both sites as the sum of the number of resting or feeding turtles observed per hour of underwater survey time. For data analysis purposes, we used the estimated size at which green turtles undergo ontogenic dietary changes on the southwestern Atlantic (45 cm CCL; Vélez-Rubio et al. 2016) to separate immature turtles in two distinct size-classes: i) "Small Immatures" (CCL < 45 cm) and ii) "Large Immatures" (CCL > 45 cm). The minimum sizes for mature turtles were defined as CCL > 80 cm for females (minimum size observed for nesting females at São Tomé island (S. Vieira, *pers. comm*)), and CCL > 90 cm for males. The cut-off size for males coincides with the minimum reproductive size estimated in the Atlantic (Goshe et al. 2010); furthermore, males captured in this study with a CCL < 90 cm did not show signs of reproductive activity, such as plastron softness or mating wounds (Wibbels et al. 1991; Blanvillain et al. 2008).

Isotopic niche parameters were computed using SIBER package V.2.0 (Stable Isotope Bayesian Ellipses in R; Jackson et al. 2011) in R V.3.2.2 (R Development Core Team, 2013). This program fits bi-variate ellipses of isotopic space using Bayesian inference to describe and compare the isotopic niche of different life-stages and or/sites. Standard Ellipse Areas (SEA)

were corrected (SEA_c) for low sample size using $SEA_c = SEA\ (n-1)(n-2)^{-1}$. Niche overlap was measured using the overlapping areas of the corrected standard ellipses of each life-stages group instead of the convex hulls, due to the small sample size (Jackson et al. 2011, Syväranta et al. 2013).

We used SIAR v.4.2 (Stable Isotope Analysis in R; Parnell & Jackson, 2013), a bayesian-mixing model that accounts for variation in isotopic discrimination and source values (Moore & Semmens, 2008), to explore the potential contributions of the most abundant groups of primary producers versus that of consumers (occupying a different trophic level) to the diets of green turtles captured at each foraging ground. Because trophic discrimination factors are not known for neritic green turtles, we used 3 different estimates of discrimination factors that together should provide robust insights into trophic interactions of turtles (Burkholder et al. 2011), estimated for (1) juvenile green turtles fed on a carnivorous diet (Seminoff et al. 2006; skin tissue: $\delta^{15}N = 2.80 \pm 0.11$ ‰, $\delta^{13}C = 0.17 \pm 0.03$ ‰), (2) herbivorous Florida manatees *Trichechus manatus latirostris* (Alves-Stanley & Worthy, 2009; skin tissue: $\delta^{15}N$ [estimated] $= 5.0 \pm 0.00$ ‰, $\delta^{13}C = 2.80 \pm 0.09$ ‰), and (3) average $\delta^{13}C$ and $\delta^{15}N$ discrimination factors based on meta-analysis of isotopic studies by Caut et al. (2009) ($\delta^{15}N = 2.75 \pm 0.1$ ‰; $\delta^{13}C = 0.75 \pm 0.11$ ‰). We ran the analysis per size-class (small vs. large immatures) as well as per location. Adults were not considered in this analysis as they are assumed to have foraged somewhere else.

RESULTS

In-Water Surveys

We recorded 95 observations of *Chelonia mydas*, in a total of 17h of combined survey time at all locations. Despite the higher number of turtles observed in FGS sites (58 and 31 turtles observed in Cabana and Ponta Santo António sites respectively, six in FGN (Ilhéu das Cabras), CPUE was similar at all sites (range 5 - 7 individuals per hour of survey time). Rough estimates of densities (as surveys were not intensive) ranged from 0.03 $ind.ha^{-1}$ at FGN and 40 - 55 $ind.ha^{-1}$ at FGS_{CAB} and FGS_{PSA} respectively. Due to the proximity of both FGS sub-sites we consider the estimated density values representative of the FGS site as a whole. Targeted efforts resulted in the hand capture of 34 individuals, including 3 males, none showed signs of being actively reproducing, and all were observed feeding before capture. One adult female captured at the northern foraging site was observed feeding on seagrass and did not show fresh mating wounds

or scars and was therefore considered as a non-breeding individual. Details and biometric parameters of turtles sampled are summarized in Table 2.

Stable Isotopes

The wide range of the values of $\delta^{13}C$ (−28.3 to −10.2 ‰) and $\delta^{15}N$ (5.8 to 13.2 ‰) observed in the animals sampled is a result of the large heterogeneity of signatures observed at the different locations, although the range of $\delta^{15}N$ values are better explained by the differences observed among different size-class groups (Table 2).

The isotopic signatures of all putative diet items are presented in Fig. 2 and on Supplementary Table 1. Macroalgae and crab items sampled at more than one location did not vary significantly in their isotopic signatures (t-test, p >0.05, n = 5 in both cases; Supplementary Table 2), therefore, the samples were pooled. As expected, all plants/ algae had a very low (and similar) $\delta^{15}N$, but their $\delta^{13}C$ varied widely, mainly because of the very low values of Rhodophytes (Fig. 2), very abundant only at the southern foraging ground.

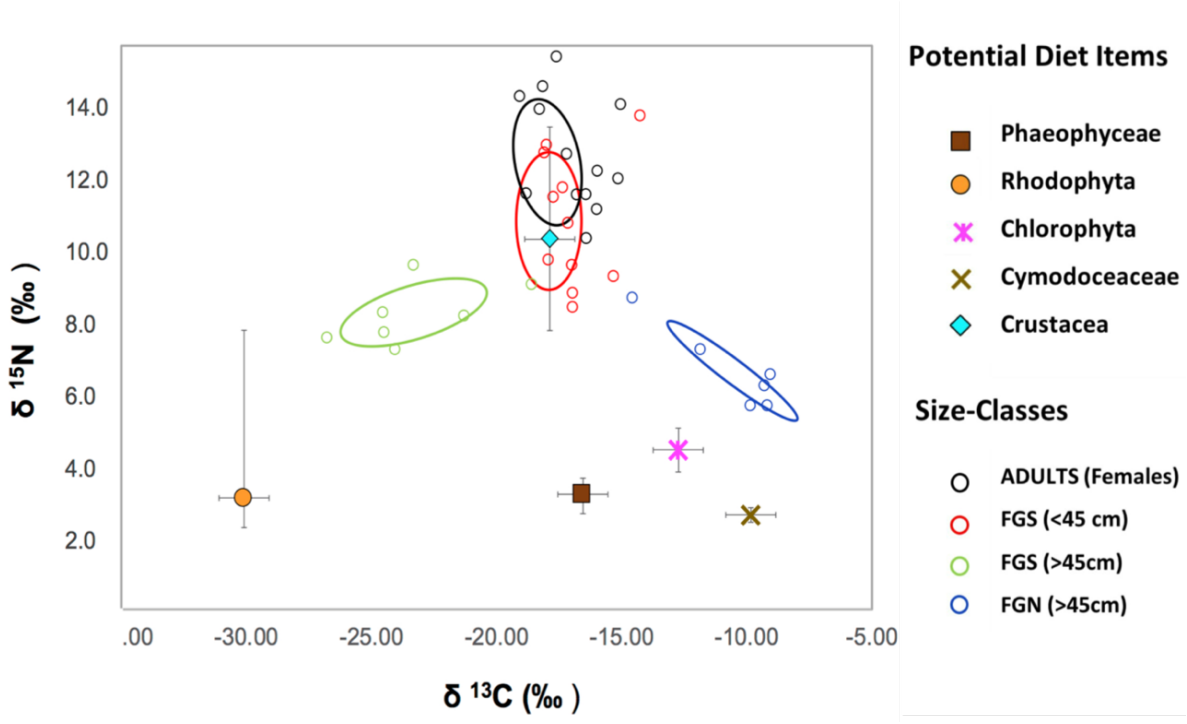

Figure 2. Standard ellipses (SEA_c) produced by SIBER indicating the trophic niches occupied by the distinct size-classes. Open circles represent individual isotopic signatures. Shaped symbols indicate mean isotopic signature of potential diet items and standard error values (bars).

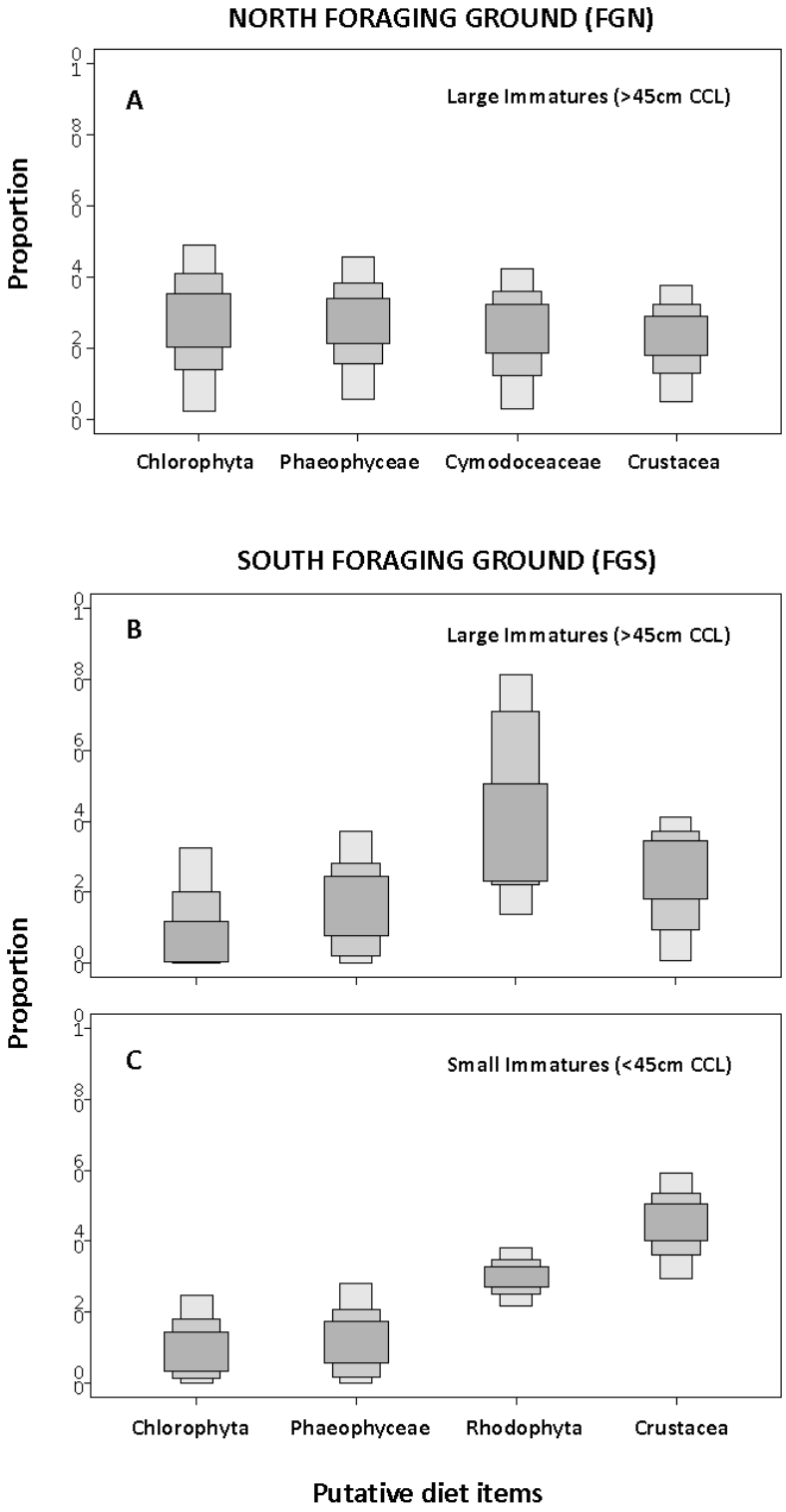

Figure 3. Potential contribution of common diet items to the diet of immature green turtles (A - large immatures at FGN; B – large immatures at FGS; C – small immatures at FGS), as determined by the SIAR mixing model.

Table 2. Summary of data obtained during in-water surveys at the two main foraging sites in São Tomé (N = number of individuals sampled).

Site	Size-Class	N	Mean CCL_{min} (cm) (min - max)	Mean $\delta^{15}N$ (‰) (min – max)	Mean $\delta^{13}C$ (‰) (min - max)
North	Large Immatures	5	73.8 ± 7.1	6.9 ± 1.3	-11.9 ± 2.3
(FGN)			(64.0 - 83.0)	(5.8 - 8.9)	(-10.2, -15.7)
	Adult (*non- breeding*)	1	109.0	6.7	-10.0
South	Small Immatures	10	38.0 ± 3.7 (34.0 - 45.0)	10.8 ± 1.8 (8.6 - 14.0)	-17.9 ± 1.2 (-19.1 - -15.4)
(FGS)	Large Immatures	8	73.0 ± 14.1 (53.0 - 87.0)	9.0 ± 1.7 (7.5 - 12.9)	-24.0 ± 3.1 (-28.3 - -19.3)
Jalé Beach	Adult Females	12	96.5 ± 5.3 (88.0 - 105.0)	12.9 ± 1.6 (10.6 - 15.8)	-18.2 ± 1.3 (-20.3 - -16.2)

The SIBER results indicate distinctive isotopic niches for each immature size-class, as well as for immatures living at each foraging ground, as the overlap among all pairs of ellipses was null (Fig. 2). Small immatures occupied an entirely different niche from the larger immatures, with their ellipse overlapping by 33 % with that of the adult females sampled at the nesting beach, and that are not supposed to forage off S. Tomé island. The distinctiveness of the larger immature's isotopic niches, and the size of their ellipses was clearly related to the two different foraging sites (Fig. 2). For this group a smaller isotopic niche was calculated for those feeding at FGN than for those at FGS (Table 3). This distinction appears to be related to the relatively high contribution of Rhodophytes (*Polysiphonia* sp.) to the diet of specimens sampled in the southern foraging ground (Fig. 3B), while none of the algae or plants at the northern site has a particular relevance to the turtle's diet (Fig. 3A). The SIAR results also suggest that animal diets may be important for immatures, especially for the small size-class (Fig. 3c, but see discussion).

Table 3. Standard Ellipse area metrics for different life-stage groups sampled in São Tomé Island

Life-stage Group	SEA	SEAc
Adults	6.52	7.03
Small Immatures FGS	6.90	7.66
Large Immatures FGS	5.93	7.12
Large Immatures FGN	2.97	3.71

Key: *SEA* standard ellipse area; *SEAc* standard ellipse area corrected

DISCUSSION

Foraging habitat use

Sea turtle fishermen indicated several foraging or aggregation sites (Fig. 1); however, we could not confirm this information in several sites, as no turtles were sighted during the snapshot surveys. Furthermore, even the number of locations provided is likely to be limited to the fishermen's experience and sites commonly used for fishing practices, and thus, biased. Nevertheless, the CPUEs and estimated densities recorded at the two selected study sites suggest that the macroalgae/ seagrass patches around S. Tomé Island, despite their small area, may maintain a few dozen sea turtles, at least during the months when the study was conducted (November - February). Considering that only two out of the several potential sites were surveyed more thoroughly and that the density of turtles in these sites was high, it is possible that future prospections will reveal more foraging grounds off São Tomé coast.

Our results show that São Tomé hosts two discreet immature groups of foraging turtles: very small immatures, likely to have recruited recently to the neritic zone from their oceanic, omnivorous life-stage, and larger immatures that explore the local resources for more extended periods, eventually as residents. The smallest turtle captured in this study was 34 cm CCL, within the expected size at recruitment range for post-pelagic turtles of this species (Musick & Limpus, 1997), and with a slightly smaller size than at other locations in the Atlantic (Reisser et al. 2013) or Pacific (Arthur et al. 2008). Small immatures were only found in the southern foraging areas; it is possible that the rocky substrate of the south of São Tomé is well suited for omnivores, being rich in macroalgae and benthic invertebrates, while providing more resting or hiding sites for the smallest individuals than the exposed seagrass beds. It can also be used

as a stopover area where green turtles recruit to after the pelagic phase and store resources before traveling to other developmental habitats (Bolten 2003, Reich et al. 2007). Nevertheless, it is possible that this size-class was not observed at the northern foraging ground due to the survey method used (e.g. lower detection of small individuals from the surface).

With the exception of one non-breeding adult female (109 cm CCL) captured in FGN, no adults were observed foraging at any of the sites during the breeding season, clearly indicating that São Tomé is an important recruitment/development habitat for juvenile green turtles in the region, and that after reaching maturity adults move to other foraging sites.

Recruitment and settlement

After recruiting to neritic habitats from pelagic waters, immatures of *C. mydas* occupy developmental habitats, which are geographically separate from both the lost-year habitat and the adult resident habitat (Carr, 1978; Meylan et al. 2011). In the developmental habitats they are expected to undergo an ontogenetic shift in foraging habits, from omnivory to feeding primarily on macroalgae or seagrass (or both) (Bjorndal, 1997; Reich et al. 2007, Arthur et al. 2008) and occupy limited home ranges associated with specific grazing areas, while feeding and growing to maturity (Makowski et al. 2006, Shimada et al. 2016). As turtles settle in a foraging area it is expected that their isotopic signatures begin to reflect those of the available diet items only after some time, since the median residence time of carbon and nitrogen stable isotopes in the epidermis of immature green turtles ranges from 27 to 35 days and from 11 to 31 days, respectively (Reich et al. 2008). There are no estimates for isotope turnover rates of large immatures, but alligator turn-over rates have been shown to be up to two years (Rosenblatt et al. 2012). As the slower growing tissues of larger immatures have longer turnover times (Martinez del Rio et al. 2009), the clear separation of the isotopic niches of turtles living at each foraging ground and the low variation in stable isotope values within each group is a strong indication of local settlement over time frames of at least many months (as in Bolnick et al. 2002, Bearhop et al. 2004, Cardona et al. 2009, Martinez del Rio et al. 2009). Residence periods of immature green turtles at several foraging sites have been estimated to be as low as 744 days in Japan (Shimada et al. 2014), 11.2 years (with a median of 2.4 years) in Brazil (Colman, 2015) (interquartile range 1.2 – 4.2 year) and up to 32 years in Bermuda (Meylan et al. 2011). Moreover, as slow-maturing animals that may take from 14 to 44 years to mature (Bjorndal et al. 2000, Bell et al. 2005, Goshe et al. 2010, Patrício et al. 2014), it is possible that these immatures remain in São Tomé waters for extended periods. Exclusive settlement to either site must however be interpreted with caution, as the small sample size at FGN may lead to an underestimation of the niche width (Syväranta et al. 2013).

Trophic status and diet preferences

Ontogenic diet shifts in green turtles from omnivory to herbivory has been thought to be abrupt and irreversible, despite growing evidence that high levels of omnivory remain amongst different life stages (e.g. Cardona et al. 2009, Burkholder et al. 2011, Lemons et al. 2011, Gonzaléz-Carman et al. 2012, Burgett et al. 2018). Should immature green turtles be primarily herbivorous, their isotopic signature should be one trophic level above the primary producers, and reflected by tissue $\delta^{15}N$ enrichment of ~2.8 ‰ (Seminoff et al. 2006). The high nitrogen stable isotopic values of all the small immatures sampled suggest high levels of omnivory prior and/or soon after recruitment to neritic habitat in São Tomé, as observed elsewhere (e.g., Cardona et al. 2009, Burkholder et al. 2011, Lemons et al. 2011, Gonzaléz-Carman et al. 2012). Even for larger immatures, the observed $\delta^{15}N$ values at both foraging grounds are higher than the expected values for strict herbivores, considering the signatures of the most common algae (Fig. 2). These animals may be supplementing their diet with animal protein (Fig.3C), or may still be far from the isotopic equilibrium with their diets. Further evidence is obtained by the inclusion of a primary consumer in the isotope mixing models. Although it is not possible to ascertain direct consumption of these specific crustaceans or any animal matter due to the limitations of our sampling approach and of the mixing models, our results suggest that the contribution of animals for the diets of immature green turtles is not negligible. Despite the omnivory suggested for all immature stages in São Tomé waters, a clear diet ontogenic shift is suggested by the contrasting signatures of small and large immatures, reflecting adjustments to a new diet.

The differences in isotopic signatures between the two groups of large immatures are mainly explained by the contrasting distributions of the red algae *Polysiphonia sp.*, that is the dominant species at the southern foraging ground, and of a seagrass, *Halodule sp*., and a green algae, *Caulerpa* sp., found mainly at the northern foraging ground. Red algae such as *Polysiphonia* have more negative $\delta^{13}C$ values than other algae, which is attributed to their photosynthetic pathways (Raven et al. 2002). The importance of the red algae mats for our results is further reinforced by the lack of spatial variation found in carbon signature of the brown algae *Dictyota* sampled at both sites. This observation is in line with other studies that show that at foraging grounds where green turtles are algal feeders, algae within the division Rhodophyta are the most commonly found in the diet (e.g. Mortimer 1981, Brand-Gardner et al. 1999, López-Mendilaharsu et al. 2008). Previous studies show that this class of algae has higher nutrient content (Montgomery & Gerking, 1980; Brand-Gardner et al. 1999), higher protein (Fleurence, 1999; McDermid & Stuercke, 2003) and digestibility (Wong & Cheung, 2001), which may be a strong factor affecting the foraging preferences of green turtles. In the North, the foraging

ground is mostly limited to the existing seagrass mats, which offers a variety of prey items, yet represents a much smaller area (estimated area of 1500 ha; Alexandre et al. 2017), when compared with the southern feeding ground, and turtles appear to be less selective in their diet.

CONCLUSION

Taking into consideration that only two of the available foraging areas were surveyed, and that the number of turtles on those two sites was high, São Tomé, as well as the similar islands on the Gulf of Guinea, may provide an important array of suitable foraging habitats for immatures of *C. mydas* in the region. There are clear evidences of settlement and local exploitation of available resources, as well as of variation in foraging behaviour between various size-classes and life-stages. These results suggest that conservation efforts should account for the possibility that subsets of the larger regional population may play different ecological roles and may be differentially vulnerable to anthropogenic impacts. Our study reveals the need for further research in neighbouring islands in the Gulf of Guinea to assess the importance of these aggregations of immature turtles to each of the regional management units identified for this populations in the Atlantic.

Acknowledgements

This study was only possible with the collaboration of local sea turtle fishermen of São Tomé island; the knowledge and experience of Aladino Santos (Didy) and Lúcio Taveira and his team, as well as the members of the Fishermen's Association of Santa Catarina, were extremely invaluable. João Santos provided diving equipment and logistical support for collection of algae and seagrass. Samples from nesting females were collected by the local research assistants working at Jalé beach, often under challenging conditions: the brothers João and Fânio, and the biologist Yaiza Pérez-Yanes. The work was conducted with the permission of the Director of the National Environment Agency of São Tomé and Príncipe, Eng. Arlindo Ceita de Carvalho. Finally, we are thankful to Ana Rita Patrício and Betânia Ferreira-Arnaud for their help reviewing the final manuscript. This study was funded by national funds through FCT - Fundação para a Ciência e a Tecnologia in the frame of the projects PEst-OE/BIA/UI0329/2014, and a doctoral scholarship awarded to the main author (PD/BD/52599/2014). All samples used in this study were collected and exported under the CITES permits 15ST00009/AC; 17ST000001/AC.

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SUPPORTING INFORMATION

Table S.1. Stable Isotope values for the putative diet items sampled

Diet Item	Mean $\delta^{13}C$	SD $\delta^{13}C$	Mean $\delta^{15}N$	SD $\delta^{15}N$
Chlorophyta	-12.74	0.62	4.52	0.62
Phaeophyceae	-17.41	0.70	3.01	2.16
Rhodophyta	-30.10	0.84	3.16	4.76
Cymodoceaceae	-9.84	0.21	2.68	0.21
Crustacea	-17.88	2.60	10.51	3.18

Table S2. Results of statistical significance tests (t-test) for different isotopic signatures observed for two diet items (crustacean *Grapsus sp* and brown algae *Dictyota sp*) collected at distinct sites on São Tomé island.

Species	Isotope	Site	N	Mean	SD	t value	p value
Grapsus sp.	$\delta^{15}N$	FGS	5	11.0	4.15	0.534	0.622
		FGN	5	10.02	2.22		
	$\delta^{13}C$	FGN	5	-17.42	3.63	0.553	0.609
		FGS	5	-18.34	1.18		
Dictyota sp.	$\delta^{15}N$	FGS	5	3.22	0.43	1.231	0.286
		FGN	5	2.96	0.23		
	$\delta^{13}C$	FGN	5	-16.62	0.48	0.775	0.481
		FGS	5	-16.76	0.58		

PAPER 3

Overcoming field monitoring restraints in estimating marine turtle internesting period by modelling individual nesting behaviour using capture-mark-recapture data

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Green turtle

Hancock, J.M., Vieira, S., Lima, H., Schmitt, V., Pereira, J., Rebelo, R. & Girondot, M. (2019). Overcoming field monitoring restraints in estimating marine turtle internesting period by modelling individual nesting behaviour using capture-mark-recapture data. *Ecological Modelling, 402,* 76-84.

Overcoming field monitoring restraints in estimating marine turtle internesting period by modelling individual nesting behaviour using capture-mark-recapture data

ABSTRACT

Marine turtles are intra-seasonal iteroparous animals; they nest from one to up to 14 times during the nesting season, laying up to 180 eggs each time. Their annual reproductive effort can therefore be estimated from clutch size, nesting frequency, and length of the nesting season. Moreover, the estimation of nesting frequency, usually obtained from the interesting period (i.e., the time in days between two nesting events) is essential for assessing the number of females in a population. However, the internesting period is strongly influenced by variation in individual behaviour of the nesting female, including abortion of nesting attempts. It is also affected by imprecise detection of females during beach monitoring, often related with a lack of fidelity to the nesting beach. Using an individual-focused model based on capture-mark-recapture data we were able to statistically characterize the nesting behaviour of the populations of green turtles (*Chelonia mydas*) and olive ridley turtles (*Lepidochelys olivacea*) in São Tomé and Príncipe (Eastern Atlantic). The developed model proposes a novel approach in estimating the internesting period, by including the different factors that lead to the heterogeneity observed in the duration of internesting periods across a single season, corrected for the probability of a female aborting a nesting process. The calculated lengths of the internesting periods for the two species are congruent with previous estimates, validating the model. A limitation of the model is its inability to estimate the true clutch frequency at the scale of the population, but it was not its purpose.

Keywords: *Chelonia mydas*; *Lepidochelys olivacea*; internesting period; iteroparity; nesting abortion

INTRODUCTION

Female marine turtles come ashore and nest several times during the nesting season at regular intervals (Miller, 1997). The number of days between consecutive clutches, named hereafter the internesting period, is typical for each species (Alvarado & Murphy, 1999). For instance, leatherback turtles (*Dermochelys coriacea*) have the shortest internesting period, typically lasting only 10 days (Fretey & Girondot, 1988), while for cheloniids this average interval spans from 12 (in green turtles, *Chelonia mydas*) up to 20 days (in olive ridley turtles, *Lepidochelys olivacea*). Several factors are thought to influence this intra-seasonal iteroparity pattern. Marine turtles, as most ectotherms, are mainly capital breeders, storing most of their energy at the foraging sites prior to their reproductive migration (Bonnet et al. 1998; Myers & Hays, 2006); the shorter the nesting season is, the less time females spend away from their foraging sites. On the other hand, when the nesting season encompasses several months, different clutches of a single female will incubate under various temporal conditions. Marine turtles are species with temperature-dependent sex determination in which sex is determined by temperature during the middle-third of the development period of the embryo, and so the distribution of clutches along several months could be also a strategy to ensure that both sexes are produced (Fuentes et al. 2017). Thus, both shorter and longer nesting seasons can be advantageous. Within each nesting season the internesting period (basically the number of days that elapses between 2 clutches (Frazer & Richardson, 1985)) is related to the time that each clutch of eggs takes to develop inside the turtle's body cavity (Miller, 1997; Rostal et al. 1996), and to the size of the cavity itself (Hays, 2001). It would be expected that the longer the internesting periods are, the more time the female has to develop more eggs and increase clutch size, reducing the number of incursions on the beach, where it is particularly vulnerable.

On every monitoring program following individually-marked females, the observed clutch frequency (OCF) is simply the number of clutches observed for a single female during the nesting season (Frazer & Richardson, 1985), and it is a key parameter in the estimation of population size (e.g. Broderick et al. 2002). However, the actual number of clutches laid by a female within a season is difficult to estimate due to imperfect capture probability, either because of fieldwork constraints or of the ability of females to choose different nesting beaches in different nesting events (Tucker, 2009, 2010).The regularity of the internesting period (*IP*) has been used to calculate the estimated clutch frequency (*ECF*): $ECF = 1 + (d_2 - d_1)/IP$ with d_1 representing the ordinal date of first observation of the nesting female in the season, and d_2 the ordinal date of last observation of the nesting female in that same season. *ECF* is thus equal to or higher than *OCF*.

Sound estimates of the interesting period are not easy to obtain for several reasons: (i) some females abort the nesting process upon emergence on the beach, not returning to nest until several days later, (ii) some females may not be detected by patrols while being on the beach, (iii) in most situations, it is not known if the female has indeed laid eggs or if it has aborted the nesting process, and (iv) fidelity to the nesting beach is not perfect. All these different events can co-occur making the estimation of the number of days between two observations difficult. For example, when a female leatherback turtle, which typically nests every 10 days (Girondot & Fretey, 1996), is seen for the second time on the beach 30 days after the first visit, it may be interpreted as its third nest after the first observation, or the second nest after the first observation if the turtle has aborted 1 or 2 nesting processes or even the first nest if the nesting process was aborted several times. It could also be the fourth nest if the internesting period of that particular female is unusually short, for example, seven days.

Until now this difficulty has been overlooked and the internesting period has been determined empirically: when a turtle is seen returning before the minimum expected IP (internesting period - for example, seven days), it is considered that the female did not lay a clutch during the first observation. Indeed, six days or less could be not sufficient for ovulation and formation of eggshells (Miller, 1997; Rostal et al. 1996), and thus two separate nesting events cannot take place within that time. If the return interval is longer than maximum expected IP (for example, 18 days), it is considered that the female has deposited one intermediate clutch that has not been observed (Frazer and Richardson, 1985).

The local NGOs Associação Programa Tatô and Fundação Príncipe Trust ensure complete monitoring coverage of all beaches in São Tomé and Príncipe islands, which is complemented by the implementation of a capture-mark-recapture program through the tagging of nesting females, providing the most complete dataset of sea turtle nest distribution in the Gulf of Guinea. These two islands host an important green turtle rookery which is genetically distinct from all others in the Atlantic (Formia et al. 2006; Hancock et al., *in press*). The second most common species is the olive ridley turtle, believed to represent a fraction of the major rookery for olive ridley in Central Africa (Girard et al. 2016). Using data obtained during the monitoring programs of these two species, we propose a novel modelling approach of the internesting period. We combine nesting counts and tagging data obtained at a rookery level to estimate this parameter, while taking into account the potential heterogeneity in the length of internesting periods resulting from female individual behavior, including abortion of the nesting process.

MATERIALS AND METHODS

Data Collection

On São Tomé island, green turtles nest mostly in the southern coast, with most of the nesting activity being concentrated between the beaches of Jalé and Cabana and also Planta; on Príncipe island, this species nests primarily in two beaches, Praia Grande and Infante, with minor nesting occurring in Boi and Ribeira Izé/Mocotó beaches. The olive ridley turtle nests only on the island of São Tomé, mostly in the north of the island, with most nesting activity concentrated along the 9 km stretch of coastline between the beaches of Juventude and Tamarindos. The importance of these beaches for each species were confirmed by early surveys conducted by Graff (1996), and they have been subjected to full monitoring every night from October through February since 2012 (olive ridley) and 2015 (green turtles). Locations are shown in Fig. 1.

Monitoring effort during night patrols was standardized at all above mentioned beaches and set to take place each night between 6 p.m. and 5 a.m. by groups of 2 trained assistants, each group covering 1.5 km stretch of contiguous coastline. During each patrol, data on female or track encounters was collected, and each female encountered was checked for metal tags, or tagged when no tags were found, to allow individual identification. Tagging was done placing a pair of Inconel flipper tags (National Band and Tag Co., Style 681) on the trailing edge of each of the fore-flippers after egg laying.

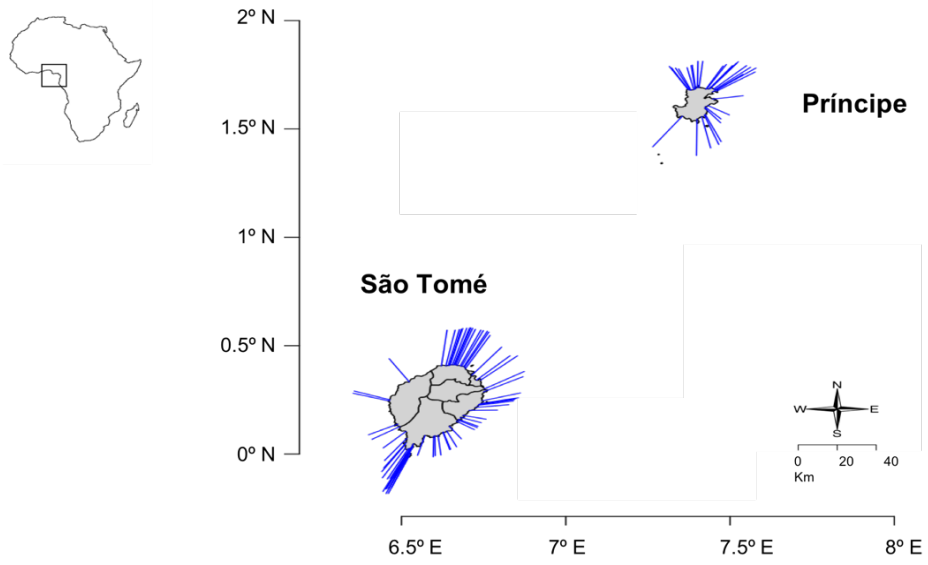

Figure 1. Map of São Tomé and Príncipe. Beaches used by marine turtles are shown with ticks (65 at São Tomé and 29 at Príncipe Island).

Data preparation and use

The data collection implemented during the monitoring program allowed the compilation of the dates of the first and all subsequent observations (re-captures) of individual females within each season on each beach. Our dataset comprised of 757 individual green turtle females (n = 1738 captures) over two seasons (2015-2016 and 2016-2017) and 635 individual olive ridley turtles (n = 700 captures) over four seasons (2012-2017). A summary of the data used is found on Table 1.

Table 1. Number of individual green (*Chelonia mydas*) and olive ridley (*Lepidochelys olivacea*) marine turtle females identified and frequency of observations between 2012-2017 in São Tomé and Príncipe islands.

Island	Season	N females	N obs.	Observation Frequencies of individual females								
				1	2	3	4	5	6	7	8	9
GREEN TURTLES *(Chelonia mydas*)												
São Tomé	2015-2016	172	336	88	36	25	16	6	1			
São Tomé	2016-2017	109	149	82	17	7	3					
Príncipe	2015-2016	355	911	133	75	48	50	27	12	6	1	3
Príncipe	2016-2017	121	342	36	24	22	14	16	7	2		
OLIVE RIDLEY TURTLES (*Lepidochelys olivacea*)												
São Tomé	2012-2013	56	57	55	1							
São Tomé	2013-2014	32	32	32								
São Tomé	2014-2015	154	173	135	19							
São Tomé	2015-2016	138	153	124	13	1						
São Tomé	2016-2017	255	285	226	28	1						

Model development

A stochastic model was formulated to describe the nesting process after the first observation of a female on a beach (Fig. 2). If the female was unable to nest and aborted, with a probability ${}_{p}Abort$, then it was expected to return for another nesting attempt after $logN(meanAbort, sdAbort)$ days. When it returned to the beach, this female would be seen with a probability ${}_{p}Capture$. After a successful nest, the female could not return to nest before the minimum interesting period, ${}_{min}IP$ (when used, ${}_{min}IP$ is an integer). Its return occurred after $logN(meanIP, sdIP) + (Nclutch - 1) \times DeltameanIP$ with N_{clutch} being the rank of the nest (i.e., 1st, 2nd, etc.). This female would produce *x* clutches (see below about parametrization

of *x*). If it was its last recorded nesting event for the season and it was successful, we considered this observation as the final one. The model is schematized in Fig. 2.

The distribution of the number of clutches per female after its first observation on the beach, *DF* (*CF* is the common acronym for Clutch Frequency, *D* rather than *C* is used in this case to indicate that it is not the true *CF*), can be obtained from a parametric model $logN(meanDF, sdDF)$. An alternative parametrization uses *DF.1*, *DF.2*, to $DF._{max}$ (*DF.0* is fixed to 1) and the probability that a female laid *x* clutches after its first observation on the beach is p_x = abs (*DF.x)*/∑abs (*DF.i)*. This parametrization has the advantage of not forcing any shape on the distribution of the clutch frequency. It is important to note that *DF* is the distribution of the number of clutches after the first observation of an individual female on the beach and therefore it is not equal to *CF,* which is the distribution of the number of clutches that a female is laying during a complete nesting season, taking into account that some females are not observed during their first nesting attempt.

This model generated a theoretical distribution of the number of observations for 0 to $_{max}Days$ with $_{max}Days$ being the maximum number of days before a recapture after the first observation. Then a set of expected number of captures C_{day} for days 0 to *m* after the initial capture was obtained (0 indicates that a female was seen twice in the same night, after aborting its first nesting attempt). These values were transformed into probabilities using $p_{day} = C_{day}/\sum C_{day}$ (Fig. 2). The larger the *N*, the closer the distribution of p_m is to the true distribution.

The development and testing of the model was performed with the green turtle data because nesting is concentrated on few individual beaches, which facilitates the full coverage of each beach by the night patrols and thus increasing the chances of observing a turtle. Olive ridley turtles nest sparsely over several kilometers of coast, reducing the chances of encounters by the night patrols; for this reason, observations are much sparser than for green turtles. We used the data collected for this species for testing and validating the model in cases when recapture rates may be lower, resulting in lower quality data. This situation is indeed frequent in marine turtle monitoring programs that suffer constraints in field data collection.

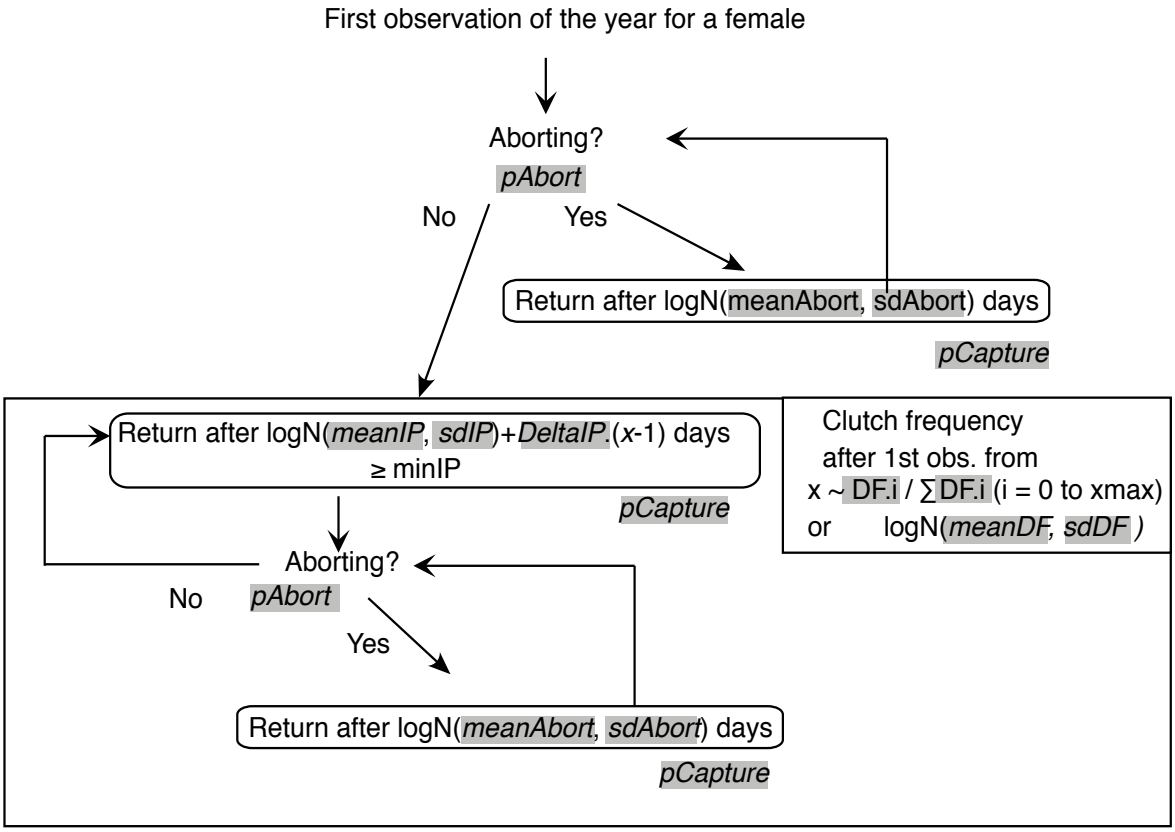

Figure 2. Algorithm of the nesting process of a marine turtle female on a monitored beach (fitted parameters are in grey boxes).

Fitting the parameters of the nesting process The data obtained from the beach monitoring (Fig. 3) was organized in k observations (*i.e.*, k individuals) in series of $n_{i.days}$ (i is the individual and *days* the number of days after first observation) with 0 (no capture) and 1 (capture). The likelihood of the observation i given the outputs p_{day} of the model is based on a multinomial distribution:

$$L_i(N_0 = n_{i.0}, \cdots, N_m = n_{i.m}) = \frac{n_i!}{n_{i.0}! \cdots n_{i.m}!} p_0^{n_{i.0}} \cdots p_m^{n_{i.m}}$$

The log likelihood of all the observations given the model is:

$$log(L) = \sum_{i=1}^{k} log(L_i)$$

with L_i being the likelihood to capture the individual i after $n_{i.0}$ to $n_{i.m}$ days. L is the likelihood of the observations for all the k individuals; in this formula, the organization level is the individual. An alternative option is to use the daily sum (top of Fig. 3) as the values for n_{days}.

In this case, the organization level is the nesting event, but the females with a larger estimated clutch frequency will have a larger impact on global likelihood than the ones with lower estimated clutch frequency. This solution has not been retained here.

The parameters $_{p}Capture$ and $_{p}Abort$ were fitted as -*logit* of the corresponding probabilities to ensure that they remained estimable at all times without defining constraints during the fit. The parameter values maximizing the likelihood were fitted using the Nelder-Mead followed by Broyden-Fletcher-Goldfarb-Shanno method with R package *optimx* (Nash & Varadhan, 2011). To test the suitability of different models fitted with the same datasets, we used the AIC estimator (Burnham & Anderson, 2002. AIC is a measure of the quality of the fit penalized by the number of parameters used, calculated as $-2\ _{log}(L) + 2\ p$ with p being the number of parameters of the model (Akaike, 1974); models with lower AIC have more chance to better represent the process that generated the data. The model has been scripted in R language and is available in the R package *phenology* (version 7.1 and above) (Girondot, 2018b).

Stability of likelihood A stochastic model was used to generate the distribution of p_{day} (see previous section). Thus, from run to run, the values change. We needed to minimize the inter-run variability of the likelihood of data given the model to ensure that a maximum likelihood fit could operate under a realistic computing time. To determine the best combination of the number of replicates and computing time, we ran the model with 10^4 to 10^5 steps (by 10^4 steps), and $2x10^5$ to $2x10^6$ (by 10^5 steps) replicates to study the dispersion of the log likelihood. For this test we used the parameters at maximum likelihood fitted using 10^6 replicates.

Identifiability of the parameters The Metropolis-Hastings algorithm is a Markov Chain Monte Carlo (MCMC) method for obtaining a sequence of random samples from a probability distribution for which direct sampling is not available or difficult (Chib & Greenberg, 1995). It was used to estimate the posterior distribution for each parameter over 10,000 iterations. This value has been chosen based on the Raftery & Lewis (1992) diagnostic. Maximum likelihood estimates were used as initial parameter values during the MCMC search, using no adaptation iteration. Proposed distributions were adapted after every 500 iterations using the method of Rosenthal (2011) as implemented in the R package *HelpersMG* (Girondot, 2018a). Priors were all obtained from a uniform distribution with limits being always very wide to ensure that a large range of parameter values could be checked (see supplementary material). Convergence was first visually examined to ensure that the time series of the parameters were stationary, and then tested using the Heidelberger & Welch (1983) diagnostic. The standard error of the

parameters was estimated after correction for autocorrelation (Roberts, 1996). Results from the MCMC were analyzed using the R package *Coda*, version 0.19-1 (Plummer et al. 2011).

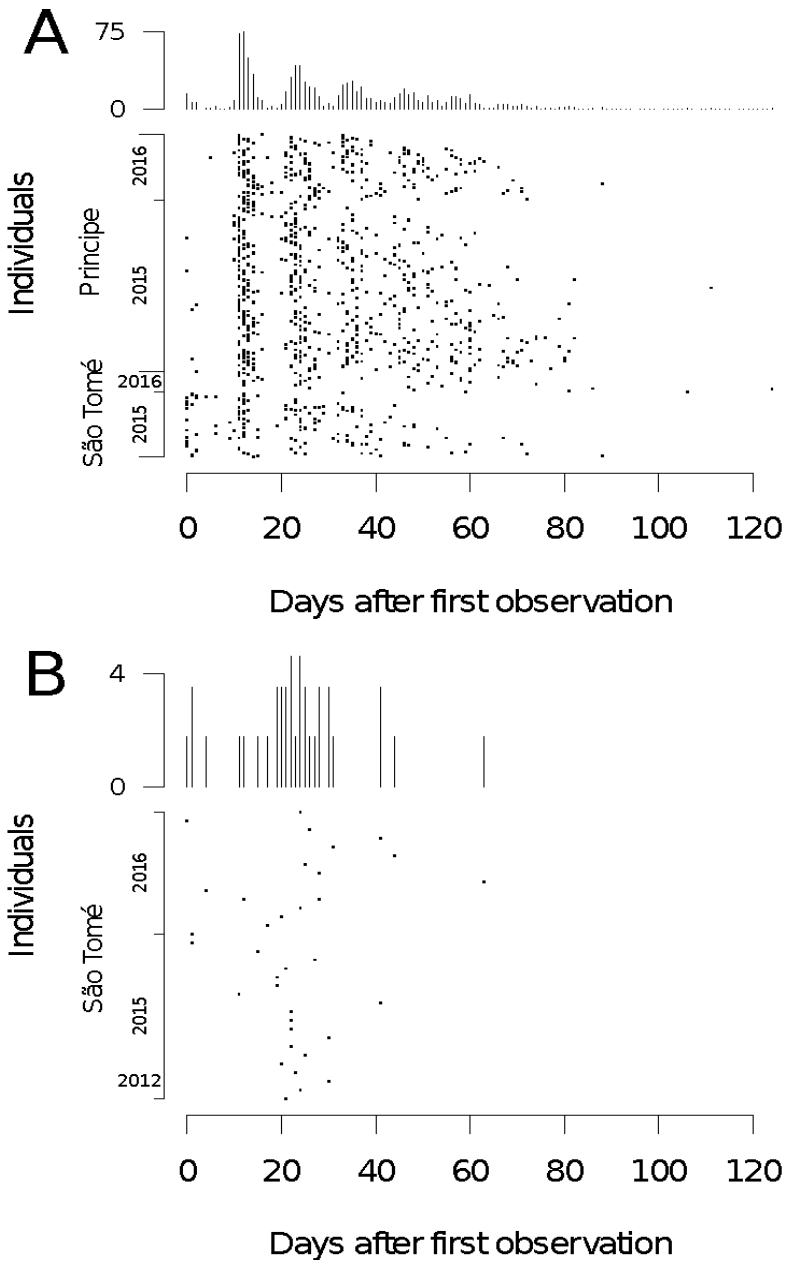

Figure 3. Distribution of individual daily observations in São Tomé and Príncipe. Rows represent the different captures of a single individual. The sums of all daily observations are depicted at the top of the figure. (A) green turtles (*Chelonia mydas*) and (B) olive ridley turtles (*Lepidochelys olivacea*).

Covariations of all parameter pairs were checked visually using bivariate plots and Pearson correlation coefficients.

The comparison between the distribution of priors and posteriors after the Metropolis-Hastings MCMC run show that some parameters cannot be estimated using this model because the posterior distribution is very similar to the prior distribution. The *DF* distribution ($_{mean}DF$, $sdDF$ or $DF_{.x}$) as well as the capture and abort probabilities ($_{p}Capture$ and $_{p}Abort$) are not identifiable. High values (> 9) of the parameter $_{min}IP$ can be excluded, but the lowest cannot. Finally, the parameters $_{mean}IP$, $_{sd}IP$, $_{Deltamean}IP$, $_{mean}Abort$ and $_{sd}Abort$ are identifiable (see supplementary material). The only very strong covariation of parameters is between $_{mean}IP$ and $_{Deltamean}IP$: their negative correlation indicates that when $_{Deltamean}IP$ tends towards 0, $_{mean}IP$ is lower (see supplementary materials).

From estimating the number of days between observations to clutch frequency By knowing the distribution of the number of days between two clutches or nesting abortions, as well as the probabilities of a turtle aborting a nesting process or being observed (captured), it was possible to relate the number of days between two observations on the beach and the true number of clutches between these two observations. A total of 10^6 simulations were performed using the green turtle fitted parameters. In each simulation, for each turtle captured we recorded the number of days after its first observation (capture) on the beach and the number of clutches observed being laid by that female up to that day. Consolidating this information on a data frame, we used it to calculate the probability that an observation of a female X days after its first observation was the n^{th} clutch.

Stability of the likelihood Likelihood calculated with 10^4 iterations was quickly estimated but the inter-run likelihood variability was too high to be used during the fitting process. On the other hand, the calculation of the likelihood with 10^6 iterations took too long to be used routinely. A number of 10^5 iterations was considered an adequate compromise as it provided a correct fit to our data (Fig. 4) and was used thereafter.

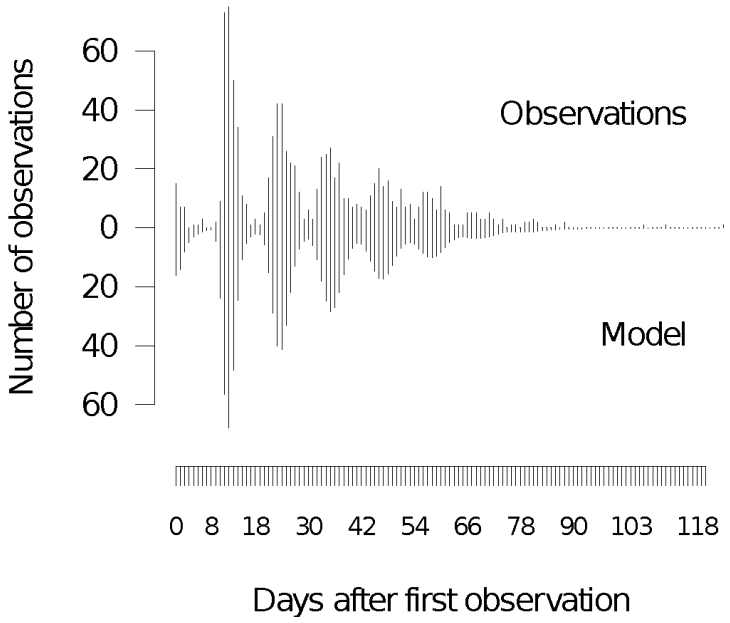

Figure 4. Comparison between observed and modelled distribution of the internesting periods for green turtles in São Tomé and Príncipe.

RESULTS

The distributions of the internesting periods for the two turtle species, considering both nesting seasons (2015-2016 and 2016-2017) and islands (São Tomé and/or Príncipe) are shown in Fig. 5. The mean shortening of the IP along the successive clutches was similar between different datasets, therefore we chose to combine these to have a global estimate for the region with the lowest confidence interval.

Green turtles Patterns of the internesting periods observed for either São Tomé or Príncipe green turtles were very similar (Fig. 3). Several peaks were observed, for 12, 24, 36 days after the first observation, and successive peaks were entangled (i.e., the lowest part of one peak distribution overlapped the highest part of the previous one). Other peaks were observed after 40 days, but they were more difficult to discriminate because the dispersion of the peaks for the higher number of days is higher, making the peaks flatter. The number of days between the first and the last observation was highly related to the ordinal date of first observation (linear model, *t*-test, $p = 0.002$); the earlier the turtle was first seen, the longer it was observed on the beach.

The green turtle mean internesting period between the first and the second clutch was estimated at 12.32 days (95 % confidence interval from 12.26 to 12.37). The 95 % range of all internesting periods was between 10.10 and 15.05 days (Table 2; showing also the values estimated for other populations). The internesting period became shorter as the number of clutches increased ($_{Deltamea}nIP$ parameters are all negative, data not shown). This effect is noticed for each of the 4 datasets, as well as when combined, and the inclusion of the $_{Deltamean}IP$ parameter greatly improved the fitting of the model (ΔAI_C = 19.76, Akaike weight p = 0.999). The fitted estimation of the minimal number of days between two clutches was 8.12 days (95 % confidence interval from 8.11 to 8.13 days). When a nesting attempt was aborted in our model simulation, the time before the next attempt was on average 1.59 days (95% confidence interval from 1.57 to 1.60) and 95% of the values were between 0.23 to 10.88 days. It should be noted that the upper 95% limit of the confidence interval (10.88 days) was higher than $_{min}IP$ (8.12 days), therefore the return to the nesting beach after an abortion event could be confused with a new clutch. The probability that a female laid a n^{th} clutch when it was recorded X days after its first observation during the season is shown in Fig. 6; Table 3 depicts the probabilities of the various clutch ranks according to the different number of days after the first observation, shown as dotted vertical lines in Fig. 6.

Olive ridley turtles Mean internesting period between two successive clutches was estimated at 22.92 days (95% confidence interval ranged from 22.85 to 23.00 days). The 95% range of internesting periods varied between 16.58 and 31.70 days (Table 2, showing also the values estimated for other populations). The change of the internesting period dependent on the progression of clutch rank ($_{Deltamean}IP$ parameter) could not be calculated due to the paucity of recapture data, and $_{Deltamean}IP$ was fixed to 0. The fitted minimum of the minimal number of days between two clutches was 9.16 (95% confidence interval from 8.37 to 9.95 days). When we simulated the abortion of a nesting attempt, the time until the next attempt averaged 3.47 days (95% confidence interval ranged from 3.23 to 3.71 days) and 95 % of the values are between 0.54 to 22.21 days. Similarly, to what was observed for green turtles, the upper 95% limit of the confidence interval (23.24 days) is higher than $_{min}IP$ (9.16 days), meaning that the return on the beach after an abortion event could be confused with a new clutch.

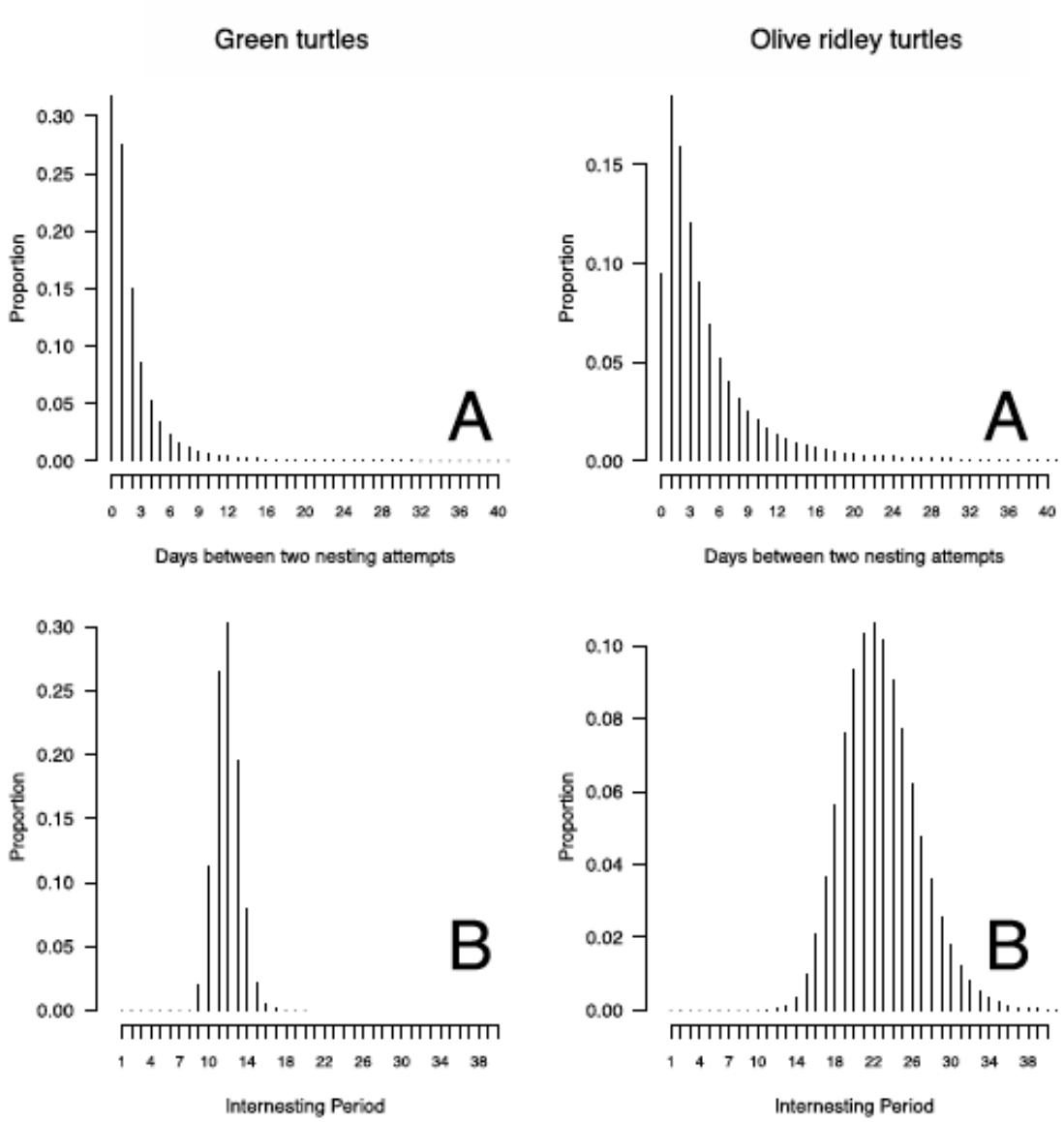

Figure 5. (A) Distribution of the number of days between two nesting attempts after a nesting abortion, and (B) interesting periods, for green (*Chelonia mydas*) and olive ridley (*Lepidochelys olivacea*) turtles.

Table 2. Internesting periods in a) *Chelonia mydas* and b) *Lepidochelys olivacea*. Min and Max represent the range of IP used to estimate mean and SD. The values in N column has a non-consistent definition across the publications: It can be the number of females, the total number of observations, or the number of observations used to estimate mean and SD. Some values were estimated from published raw data (see notes).

Location	RMU	Mean	SD	Min	Max	N	Reference
GREEN TURTLES *(Chelonia mydas)*							
São Tomé and Príncipe	**E Atlantic**	**12.32**	**1.365**	**10**	**15**	**1842**	**This study**
Ascension Island	E Atlantic	13.9	2.4	7	20	840	Mortimer &Carr (1987)
Cyprus	Mediterranean	12.5	1.65	9	19	205	Broderick et al. (2002)
Costa Rica	SW Atlantic	12.1	1.64 [a]	7	18 [d]	4654	Carr et al. (1978)
Florida, USA	NW Atlantic	12.9	1.59	10	19	165	Johnson & Ehrhart (1996)
Surinam	S Atlantic, S. Carib	13.27 [c]	1.24 [a]	11	16	601	Schulz (1975)
Tromelin Island	SW Indian	12.62 [a]	1.92 [a]	8	19	3036	Le Gall et al. (1987)
Hawaii (1974)	NC Pacific	13.2	1.38 [a]	11	18	74	Balazs (1980)
Australia	SC Pacific	14.1	1.65	9	21	264	Limpus et al. (1984)
Malaysia	SC Pacific	10.5	1.33 [a]	8	17	5417	Hendrickson (1958)
OLIVE RIDLEY TURTLES *(Lepidochelys olivacea)*							
São Tomé Island	**Atlantic, East**	**22.92**	**4.39**	**16**	**31**	**415**	**This study**
India	Indian Ocean	22.09	0.58	20	25	4411	Tripathy and Pandav (2007)
Gabon	Atlantic, East	17.5	4.3	9	25	18	Maxwell et al. (2011)
Brazil	Atlantic, West	22.35	7.01	21.2	23.5	143	Matos et al. (2012)
Costa Rica	Pacific, East	24.5	7.1	14	50	33	Dornfeld et al. (2014)
Colombia	Pacific, East	18.8	4.2	16	25	4	Barrientos-Muñoz et al. (2014)

[a] Value was not calculated in the original publication, [b] Published value was 13.2 days, [c] Published value was 13.4 days, [d] The min and/or max values have been calculated to reflect the published mean value

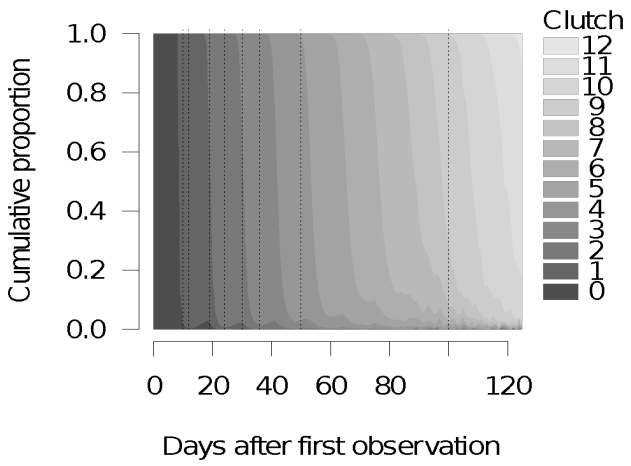

Figure 6. Probability distribution of the rank of a clutch according to the observed 90nteresting period for green turtles (*Chelonia mydas*) in São Tomé and Príncipe nesting beaches (2015-2017). The values for 90nteresting periods shown as dotted lines are summarized in Table 3.

Table 3. Clutch rank probability according to an observed internesting period (IP) for green turtles (*Chelonia mydas*). The periods considered on the first column are depicted in Figure 6 as dotted lines.

Internesting Period (days)	**Clutch Rank**										
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
6	1.000										
10	0.017	0.983									
12	0.003	0.997									
19	0.025	0.578	0.397								
24		0.007	0.992								
30	0.002	0.026	0.658	0.314							
36		0.001	0.015	0.984							
50		0.001	0.003	0.025	0.959	0.013					
100				0.001	0.001	0.019	0.023	0.049	0.193	0.710	0.004

DISCUSSION

For decades, the number of nests counted during a nesting season was converted to the number of nesting females using the formula (number of nests) / (clutch frequency) (Gerrodette & Taylor, 1999). The estimation of total number of nests during a nesting season has received general solutions (Girondot, 2010; Girondot, 2017; Girondot & Rizzo, 2015) and it can be considered as being a solved problem for most of the situations. On the other hand, a general procedure for the estimation of the number of nests per female (clutch frequency) is still needed (Briane et al. 2007). The most common procedure used the formula $ECF = 1 + (d_2 - d_1)/IP$ with d_1 representing the ordinal date of first observation of the nesting female in the season, and d_2 the ordinal date of last observation of the nesting female in that same season and *IP* being the internesting period (Frazer & Richardson, 1985). Estimation of mean *IP* is then done by averaging the number of days between all consecutive nesting attempts. However, the actual number of clutches laid by a female within a season is not known due to imperfect capture probability, either because of fieldwork constraints or of the ability of females to choose different nesting beaches in different nesting events (Tucker, 2009, 2010). Therefore, we can never be sure that two observations of the same nesting female on the beach refer to consecutive nesting events, or if some were missed. In consequence, the quality of the *IP* estimate is dependent on our ability to count the true number of nests deposited by a female, which may vary from female to female, and is nearly impossible to know. In a general move in ecology from pattern to process (Swihart et al. 2002), the estimate of such an important parameter cannot be based simply on very strong untestable assumptions. This move is particularly relevant for species with a complex life cycle such as marine turtles, for which the interpretation of changes in numbers in terms of population mechanisms is quite challenging.

The identification of a high number of individual females allowed us to observe the typical pattern of succession of peaks at multiples of 12 days which is typical of green turtles (Fig. 3). The broadening of the peaks observed in longer returns is likely due to two phenomena: the variability of the internesting periods and the fuzziness resulting from the high rate of nesting abortion classically observed for this species (Mortimer & Portier, 1989). This pattern was less clear for olive ridley turtles and it was impossible to clearly identify peaks within our data (Figure 3); however this result is particularly important because it showed that the internesting period can be evaluated even when data are sparse, as for olive ridleys.

The estimation of the internesting interval for both studied species was very reliable according to the diagnostic tools used, showing that the design and implementation of an individual-focused statistical model was successful at producing a robust estimate of the internesting period of female marine turtles. These internesting periods are likely to be dependent on the turtle's physiological reproductive capacity as well as on local external, primarily anthropogenic, factors that may disturb turtles attempting to nest (Tiwari & Bjorndal, 2000). The observation that the nesting season is longer for early nesters has also been noted for leatherback turtles in French Guiana (Fretey & Girondot, 1989). Two non-exclusive explanations were proposed: either the turtles arriving first in the nesting site laid a higher number of clutches or, most likely, the turtles that are seen first later in the season have already nested but were not observed.

Moreover, our model shows an important advance in estimating the rank of a clutch in relation to the date of the first observation of a turtle on the beach (Fig. 6), with a particularly high probability of success when the interval of days is small and close to a multiple of the mean internesting period. For example, for green turtles, when a female is observed on the beach after 12 and 24 days, the probability that these nests correspond to its second and third clutches are respectively 0.997 and 0.992 (Table 3). If the number of days is not a multiple of the internesting period, then the rank of the clutch is uncertain: for an observation 19 days after the previous observation, the probability that it is the second or third clutches are 0.578 and 0.397 respectively (Table 3). When the number of days increase, surprisingly, the determination of the rank of the clutch did not degrade too much. For example, if a female is observed after 100 days, the probability that it is her 10^{th} clutch is 0.71 (Table 3).

Another interesting result from our model, is that we demonstrate that for green turtles, the internesting period declines as clutch rank increases. The inverse relation between internesting period and clutch rank was also demonstrated in loggerhead turtles using data from intensive field work (Limpus, 1985). It is tempting to link this decrease of the internesting period with the lower number of eggs present in the clutches of higher rank as shown in loggerheads (Limpus, 1985), but testing this hypothesis with our dataset was not possible.

CONCLUSION

The use of capture mark recapture (CMR) studies on nesting beaches can be used to estimate the minimum number of reproductive turtles in each season but interpreting the nesting history of a female is a prerequisite to be able to convert an observed total number of clutches into an estimate of the number of females in a population. We consider that up to now, no model is yet able to correctly convert a dataset of observed or estimated clutch frequency (OCF and ECF) into a number of females in a population, as the impracticality of assessing this parameter is directly due to field constraints and to the variability in female behaviour. The common restraints posed by incomplete datasets that include extended time intervals between individual re-observations is solved by our model, which can be used to determine with high probability the rank of an observed clutch since the first observation. Moreover, our model demonstrates the usefulness of CMR datasets in understanding patterns in the individual behaviour of a female on the beach and how these affect the variation in internesting periods for a given population.

Acknowledgements

We thank all the beach monitoring staff of Marapa in São Tomé and Fundação Príncipe Trust in Príncipe island for their hard work at night protecting the turtles, nests and gathering the data that made these analyses possible. We also thank the authorities of São Tomé and Príncipe Republic to have authorized this research, HBD Princípe Sustainable and Responsible Development for supporting FPT activities on Príncipe Island during the time of this study. This study was partially funded by national funds through FCT - Fundação para a Ciência e a Tecnologia in Portugal through a doctoral scholarship awarded to JM Hancock (PD/BD/52599/2014).

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CHAPTER 3

TARTARUGA TATÔ

OLIVE RIDLEY SEA TURTLE

Lepidochelys olivacea

PAPER 4

Genetic diversity, multiple paternity and dispersal in an olive ridley (*Lepidochelys olivacea*) rookery in São Tomé island, West Africa

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Martinho Alves Rosa (Micoló)

"Love makes even a turtle smile."

Henrietta Newton Martin

In preparation

Genetic diversity, multiple paternity and dispersal in an olive ridley (*Lepidochelys olivacea*) rookery in São Tomé island, West Africa

ABSTRACT

The olive ridley (*Lepidochelys olivacea*) is the most abundant sea turtle (Pritchard, 1997) and has a widespread distribution. In West Africa, its main rookery is located in Gabon, followed by the islands of the Gulf of Guinea, which include the island of São Tomé, where this species has been heavily exploited in the last decades. In this study we assessed the levels of multiple paternity within the São Tomé rookery using microsatellite data obtained from females and their offspring to reconstruct male genotypes and estimate the operational sex-ratio and understand the role of adult males in promoting gene flow in the Gulf of Guinea. We further investigated if this population could be considered at equilibrium or whether it has undergone a perturbation (e.g., bottleneck or demographic expansion), and estimated genetic effective population size (N_e). Results suggest some levels of polyandry, with a male-skewed operational sex ratio of 1F : 3M and male-mediated gene flow at this rookery. Despite the potential benefits of polyandry and male-mediate gene flow to genetic diversity, the low effective population size and the evidences of a genetic bottleneck suggest that geneflow is limited and possibly confined within the Gulf of Guinea islands. We discuss these findings in light of population dynamics of this species in the Atlantic.

Keywords: operational sex ratio; paternal assessment; *Lepidochelys olivacea*; Gulf of Guinea; Eastern Atlantic

INTRODUCTION

The olive ridley (*Lepidochelys olivacea*) is the most abundant sea turtle (Pritchard, 1997) and has a widespread distribution. It is thought that the Atlantic population proceeded from the Indian Ocean and colonization started along the West coast of Africa, followed by dispersal across the Atlantic Ocean to South America (Bowen et al. 1997), where nesting has been recorded on the coast of South America and in the Caribbean. The largest rookery in the Atlantic is found in Gabon (Metcalf et al. 2015), on the West coast of Africa, with other minor nesting sites spreading from Senegal to South Africa (Biles et al. 2006; Weir et al. 2007; Tomás et al. 2010; Fretey et al. 2012). Low $_{mt}$DNA haplotype diversity was recorded in Atlantic nesting sites (with one haplotype observed in 94% of samples) (Bowen & Karl, 2007), which coupled with an overall low nucleotide diversity, and a shallow $_{mt}$DNA phylogeny relative to other Cheloniid sea turtles suggests that the colonization of the Atlantic was recent. (Bowen et al. 1997). This scenario of low genetic diversity, as it is often observed in natural populations, is generally associated with negative effects such as inbreeding depression, loss of evolutionary potential, and the accumulation of deleterious mutations (Frankham & Ralls, 1998; Frankham, 2010). These effects theoretically increase extinction risk and are expected to be stronger in populations under anthropogenic or natural stresses (Spielman et al. 2004). Because this species is almost exclusively a mainland nester, the nesting aggregations in the Gulf of Guinea islands (Bioko and São Tomé) are particularly interesting to study; these island populations may have benefited from less predation until human colonization, but since then have been subject to intensive exploitation for human consumption (Castroviejo et al. 1994) and are thus more susceptible to the threats described above.

The study of mating systems has become an increasingly useful tool to assist in understanding the processes that may affect the genetic make-up of a population and assess its level of susceptibility to change. The reproductive strategy of a species, particularly when polyandry occurs, can affect the intensity of sexual selection (Fleming & Gross 1994; Evans & Magurran, 1999), the genetic variability and introgression within a population, and finally the genetic effective population size (Ne) and the evolutionary potential of that species (Wright, 1931; Frankham, 1995; Vucetich et al. 1997; Charlesworth, 2009; Sugg & Chesser, 1994). Molecular parentage-based approaches to study mating systems are particularly appropriate in highly mobile species such as marine turtles, as mating is rarely observed, and their high vagility limits access to the animals. Sibship reconstruction from neutral genetic markers makes it possible to determine family structure even when it is not possible to sample candidate parents (e.g. Wang 2004; Wang & Santure 2009), and this approach has been used to infer the mating system (e.g.

Kanno et al. 2011; Clark et al. 2014; DiBattista et al. 2008) and estimate effective population size (Liu & Ely 2009; Li et al. 2013). Moreover, this approach allows the indirect sampling of the male component of a population of breeding turtles (Wright et al. 2012; Lasala et al. 2013; Stewart & Dutton, 2011, 2014), a great advantage since males rarely come ashore and are difficult to capture at sea. The demographic viability of a population is typically based exclusively on female fecundity, survival, and abundance, assuming that these parameters are similar for males, or that in males they simply do not matter. This leads to a conservative perspective that the number of males present in a population is of little interest provided all females are mated. However, the degree of polyandry (i.e. females mating with multiple males) can influence population-level processes, such as population growth rate and extinction risk, by altering genetic variability, the level of inbreeding, and adaptive potential (Frankham, 2005). Population structure in sea turtles is fundamentally promoted by females' natal homing behaviour or philopatry (return of adults to their natal beaches) and site fidelity (precision with which they return to the same beach in subsequent years) to nesting beaches, while genetic variability is promoted by a presumed lower level of fidelity of breeding males to courtship areas, which promotes gene flow among groups of individuals that breed in geographically distant locations. Polyandry can also have individual-level effects by altering average offspring viability and reproductive success (Treguenza & Weddel, 2002; Byrne & Whiting, 2011). Mating system dynamics may also influence a population's vulnerability to harvest as there may be upper and lower thresholds in a population's sex ratio which, when exceeded, result in reproductive collapse (e.g. Hard et al. 2006).

Genetic analyses of nesting females and their offspring can both identify the number of sires per clutch and provide data on the number of breeding males and females, from which operational sex ratios (OSRs) can then be calculated (Stewart & Dutton, 2011; Wright et al. 2012a, 2012b). Microsatellites are ideal for these studies because of their abundance, high polymorphism content, codominance, easy detection, and transferability among studies (e.g. Dawson et al. 2013). Moreover, given the low evolutionary rate of the mitochondrial genome of turtles (Avise et al.1992) and the general low haplotype diversity for $_{mt}$DNA Control region within the Atlantic Ocean for *L. olivacea* (Bowen et al. 1997), genetic diversity and demographic aspects for Atlantic populations are better assessed using microsatellite data. We compiled a microsatellite dataset for the olive ridley marine turtle population nesting in São Tomé island, (Gulf of Guinea, West Africa), a small nesting assemblage within the Eastern Atlantic Ocean regional management unit (Wallace et al. 2010) where this species was legally and heavily exploited for human consumption until 2014. We aim to assess the levels of multiple paternity within the rookery using microsatellite data obtained from females and their

offspring and to use the levels of multiple paternity to estimate the operational sex-ratio and role of adult males in promoting gene flow in the Gulf of Guinea. Finally, we use microsatellite loci to investigate if this population could be considered at equilibrium or whether it has undergone a perturbation (e.g., bottleneck or demographic expansion) and discuss implication of the results in light of population dynamics of this species in the Atlantic.

MATERIAL AND METHODS

Sample collection and DNA extraction

Lepidochelys olivacea samples were collected on a 7 Km stretch of coast on the north of the island of São Tomé, extending from Tamarindos (0°24'31.6"N, 6°38'37.6"E) to Juventude (0°23'25.8"N, 6°41'42.4"E) beaches, during the nesting seasons (October through March) of 2015-2016 and 2016-2017 (Fig. 1).

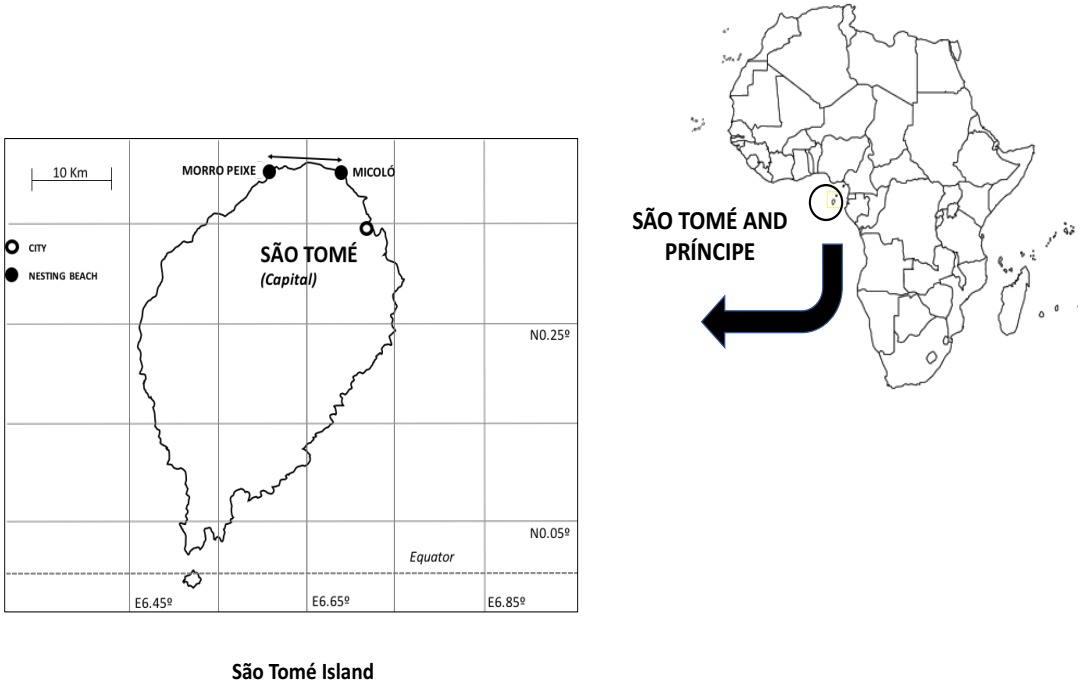

Figure 1. Location of the study site in the island of São Tomé, a 7 Km stretch of coastline comprised between the communities of Morro Peixe and Micoló, surveyed and sampled between 2015-2017.

We sampled adult females at night during beach patrols conducted by Programa Tatô's staff. Tissue samples were collected from the rear flipper of females using disposable sterile surgical scalpels. Nests of each female were relocated to an *in-situ*, fully enclosed hatchery, and

monitored daily after 45 days of incubation. Up to 20 hatchlings were randomly selected per nest and tissue samples were taken from the trailing edge of the rear flipper (approximately 2 mm^2 of tissue), using disposable sterile surgical scalpels. Ethanol was then applied to the flipper to prevent bleeding, and hatchlings were held for observation before release. This procedure was always performed by night, to ensure that that hatchlings oriented normally and crawled actively down the beach and into the sea. All tissue samples were preserved in 96% ethanol and stored at room temperature until DNA extraction. DNA was extracted using the Easy Spin kit (Qiagen Inc., Valencia, CA) following standard DNA extraction protocols.

Sequencing and microsatellite genotyping

For PCR amplification and sequencing of the CR fragment, we used the primers LCM15382/H950 developed by Abreu-Grobois et al. (2006). Thermal conditions for amplifications consisted of 15 min at 95° C, followed by 40 cycles of 30 sec duration each at 56°C, 45 sec at 72ºC with a final extension at 60° C for 20 min. Successful amplifications were enzymatically purified, and sequenced following the BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems). Sequencing products were separated in the same automatic sequencer ABI3130*xl* Genetic Analyzer and were aligned and compared in the software SEQSCAPE v.3.0 (Applied Biosystems).

Nuclear genetic variability was investigated using 14 microsatellite loci previously developed for *Caretta caretta* (Cc5H07, CcP1F09, CcP2F11, Ccp7C06, Ccp7D04, CcP7F06, Cc1F01, Cc2H12, Cc5C08, Cc8B07, Cc7C04, Cc1G02, Cc1G03, Cc7G11, Shamblin et al. 2007, 2009). Microsatellite amplifications were conducted in a Biorad T100 thermocycler using a Multiplex PCR Kit (QIAGEN) following manufacturer's instructions. The fourteen microsatellite loci were tested and amplified separately and then combined in three multiplex reactions for the final amplification using the Multiplex Manager v.1.2 software. General thermal conditions comprised an initial denaturation for 15 min at 95°C, followed by an additional step at 95°C for 30 sec., followed by 21 cycles of 1 min 30 sec. duration, each at 60°C with -0.5ºC decrease per cycle (to ensure an optimal annealing temperature for each primer). A second round of equal number of cycles was programmed at a lower, constant temperature (54ºC), set for 1 min each, to exponentially increase the number of amplified fragments. A final extension at 72°C was programmed for 35 min to promote adenylation and to avoid -A peaks during genotyping. PCR products were separated by capillary electrophoresis on an automatic sequencer ABI3130*xl* Genetic Analyzer (AB Applied Biosystems). Fragments were scored against the GeneScan-500 LIZ Size Standard using the GENEMAPPER v.4.1 (Applied Biosystems) and manually

checked twice. An individual's genotype for a given multiplex was not used in downstream analyses if more than six loci (out of the 13) from that multiplex failed to amplify, and individuals were removed entirely if more than ten loci failed in total. The presence/absence of large allele dropouts and null alleles was determined using the software MICROCHECKER v.2.3 (Van Oosterhout et al. 2004).

Paternal assessment and male genotype reconstruction

We used the program COLONY v.2.0 (Wang & Santure, 2009) to assess the levels of multiple paternity. This software assigns sibships and parentage based on a maximum-likelihood model. Offspring are clustered by full-sib and half-sib (maternal and paternal), and parent–offspring relationships are determined, with parents assigned to full-sib groups. COLONY was set to the default parameters, a single medium-length run, with full-likelihood analysis, assuming polygamy for both males and females, and performed with and without maternal genotypes. Per-locus estimates of genotyping error was set at 0.01 (Phillips et al. 2013) and the program was allowed to update allele frequencies during the analysis. Hatchlings that could not be assigned to a sampled female or a single nest were not considered in further analysis. COLONY also performs the reconstruction of the genotypes of unsampled parents on a locus-by-locus basis and provides a confidence value for each reconstruction (Wang, 2004; Wang & Santure, 2009). When reconstructing multi-locus male genotypes, we only incorporated single-locus genotypes with confidence of ≥0.90 and only used them in downstream analyses if they contained ≥ 4 of all used loci and were reconstructed from ≥10 offspring (see Phillips et al. 2013). This reduces the risk of biasing estimates of heterozygosity, since heterozygous male genotypes require fewer offspring for confident reconstruction than homozygous genotypes.

Population data analyses

Standard summary statistics for mitochondrial diversity, including the number of haplotypes (H), haplotype diversity (H_d), and nucleotide diversity (π) were calculated in the software DnaSP 5.0 (Librado & Rozas, 2009). Similar sequences were searched for using a blast search on the GenBank database (National Center for Biotechnology Information, USA: NCBI Home page http://www.ncbi.nlm. nih.gov). Departures from Hardy–Weinberg expectations (HWE) and linkage disequilibrium (LD) among the 13 loci were assessed using GENEALEX 6.503 (Peakall & Smouse, 2012) with levels of significance for HWE being adjusted using the Bonferroni correction. The same software was used to estimate the mean number of alleles per

site, the average observed (H_o) and expected (H_e) heterozygosity over loci, as well as H_o and H_e per loci per site. The same parameters were tested posteriorly using inferred male genotypes (see "***Paternal assessment and male genotype reconstruction***" below), as well as the estimated proportion of genetic diversity distributed within the adult population (females and inferred males), performing an analysis of molecular variance (AMOVA) in GENEALEX.

We estimated N_e from our full dataset (females and hatchlings) using the software Ne ESTIMATOR v.2.1 (Do et al. 2014), which estimates contemporary effective population size (N_e) using multilocus diploid genotypes from population samples applying two methods that require only a single sample: (a) the linkage disequilibrium (LD) method (Hill, 1981) and (2) the heterozygote excess method (Pudovkin et al. 1996). Mating system was set to random. The heterozygote-excess method estimates the effective number of breeding parents (N_{eb}) with no bias and fair precision when the sample size of progeny is of infinite N and when gametes combine completely at random, i.e., when all male gametes have an equal probability of combining with all female gametes, as in some polygamous, random-mating species.

We used the programme BOTTLENECK v.1.2.02 (Cornuet & Luikart, 1996) to test for genetic evidence of past changes in effective population size compared to theoretical expectations based on a population at equilibrium. This program compares a sample's heterozygosity (H_e) at each locus with that expected under mutation-drift equilibrium (H_{eq}). Heterozygosity excess ($H_e > H_{eq}$) suggests a population contraction (i.e. a bottleneck), whereas a heterozygosity deficit suggests a population expansion (Cornuet & Luikart, 1996). We used a two-phase mutation model (TPM), considered an adequate model for sea turtle studies (Hoekert et al. 2002), setting the parameters recommended by the programme's authors (non-stepwise = 5%, variance = 12). The significance of heterozygosity excess was calculated by running a Wilcoxon sign-rank test.

Sex-biased dispersal

As the direct observation of sea turtle dispersal in our study site is logistically impossible, we conducted two methods to test hypotheses regarding differential dispersal between the sexes: (a) relatedness between individuals and (b) mean assignment index and its variance, both using GENALEX v.6 (Peakall & Smouse, 2006). First, a relatedness-based test was performed to compare mean relatedness *r* (Queller & Goodnight, 1989) of female–female and male–male dyads. A significant result would suggest that the sex with the lower average relatedness is the major disperser (as dispersal increases, one expects to find fewer relatives within a given area, e.g. Prugnolle & De Meeus, 2002). To aid interpretation of the relatedness test, we ranked all

dyads by *r*, including male–female dyads, and calculated the proportions of each dyad class above increasing thresholds of *r*. If one sex is less dispersive than the other, one would predict that that sex should account for a disproportionately large share of 'higher relatedness' dyads (e.g. half-sibs ($r \approx 0.25$), full sibs ($r \approx 0.5$), and equivalents) (Phillips et al. 2013). If sex-biased dispersal is present, we expect that the average relatedness of the dispersing sex would be lower than the mean relatedness of the non-dispersing sex.

Sex-biased dispersal was also examined using assignment indices (Mossman & Waser, 1999). The assignment index calculates the probability that a particular genotype should be present in the population from which it was sampled, after correction for population differences (Goudet et al. 2002; Prugnolle & De Meeus, 2002). The corrected assignment indices (AI_c) are distributed around a mean of zero, and since recent immigrants tend to have lower AI_c values compared to residents, the dispersing sex is predicted to exhibit a lower mean AI_c compared to the more philopatric sex. Likewise, the dispersing sex should display greater variance in AI_c because it should comprise of both resident (positive values) and immigrant (negative values) individuals. We used GENALEX to calculate individual AI_c values, with the permises that the sex with the lower index value is the more dispersive. A *t*-test was used to assess the significance of sex-specific differences in the mean and variance of AI_c value. Because of method restriction, we only used genotypes with no missing data.

RESULTS

Paternal assessment and male genotype reconstruction

A total of 42 clutches (approximately 20 per season) with an average of 15.7 ± 5.3 hatchlings sampled per clutch were analyzed. Only 31 nests and 487 hatchlings could be correctly assigned to the sampled female and used with confidence for the paternal assessment, resulting in the reconstruction of 62 male genotypes. A total of 39 individual males were allocated to the assigned nests, resulting in an observed operational sex ratio of 1F : 1.3M. Only 34 males had multi-locus genotypes that met our confidence criteria (genotype with $p \geq 0.9$, at > 6 loci, reconstructed from ≥10 offspring), a result from either mating to unsampled females with single-paternity nests (n = 4) or because they did not father sufficient offspring (n = 7). Multiple paternity was observed in 32.3% of the nests (n = 10), with nests being sired by either two (n = 8), three (n = 1) or four males (n = 1) (Fig. 2).

Genetic diversity and population analyses

A total of 118 adult females were sequenced for this study resulting in the detection of one single haplotype (F). Approximately half of those females (58) and 741 hatchlings were successfully genotyped at 14 loci. No evidence of allele dropouts was observed; the locus Cc5H07 showed excess of homozygotes for most allele size classes, suggesting the presence of null alleles, and was removed from all analyses. Allele frequencies at each locus were within expectations of Hardy-Weinberg equilibrium ($p > 0.05$) and showed no significant linkage disequilibrium after applying the Bonferroni correction. All loci showed polymorphism, ranging from 6 to 16 alleles per locus across all samples, with overall levels of $H_0 = 0.732$ and $H_e = 0.728$ (Table 1).

Table 1. Summary statistics of genetic variation at 13 microsatellite loci in *Lepidochelys olivacea* population (females and offspring).

Locus	N	N_a	N_e	I	H_o	H_e	F
CcP1F09	799	6.000	2.034	1.034	0.503	0.508	0.010
CcP2F11	796	10.000	6.257	1.967	0.796	0.840	0.052
Ccp7C06	799	16.000	7.227	2.305	0.757	0.862	0.121
Ccp7D04	799	13.000	6.272	2.074	0.807	0.841	0.040
CcP7F06	799	14.000	4.794	1.840	0.796	0.791	-0.006
Cc1F01	797	13.000	6.495	2.103	0.836	0.846	0.012
Cc2H12	798	13.000	6.271	2.006	0.871	0.841	-0.036
Cc5C08	798	15.000	8.425	2.334	0.919	0.881	-0.042
Cc8B07	799	3.000	1.188	0.321	0.166	0.158	-0.053
Cc7C04	798	13.000	7.843	2.224	0.909	0.872	-0.041
Cc1G02	796	15.000	8.595	2.349	0.862	0.884	0.025
Cc1G03	796	3.000	1.998	0.700	0.543	0.500	-0.086
Cc7G11	797	10.000	3.185	1.516	0.655	0.686	0.045
Adult Population	103	9.538 (± 0.783)	5.354 (± 0.512)		0.732 (± 0.041)	0.728 (± 0.040)	-0.008 (± 0.015)

Key: Sample Size (N), number of alleles (N_a), number of effective alleles (N_e), Information Index (I), Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), and Fixation Index (F).

Using the Full Likelihood method implemented in N_e estimator, effective population size (N_e) was estimated at 53 to 57 individuals, and at 139 breeding pairs (N_{eb}). A population whose effective size has remained constant in the recent past is expected to show an approximately

equal probability of excess or deficit in the gene diversity of a locus. The TPM mutation model that we used to assess putative demographic perturbations indicated that nine of the 13 microsatellite loci had a significant excess in expected heterozygosity. These results are indicative of a significant departure from demographic equilibrium and indicate that this population has undergone a bottleneck (Wilcoxon significance rank test; $p = 0.0026$).

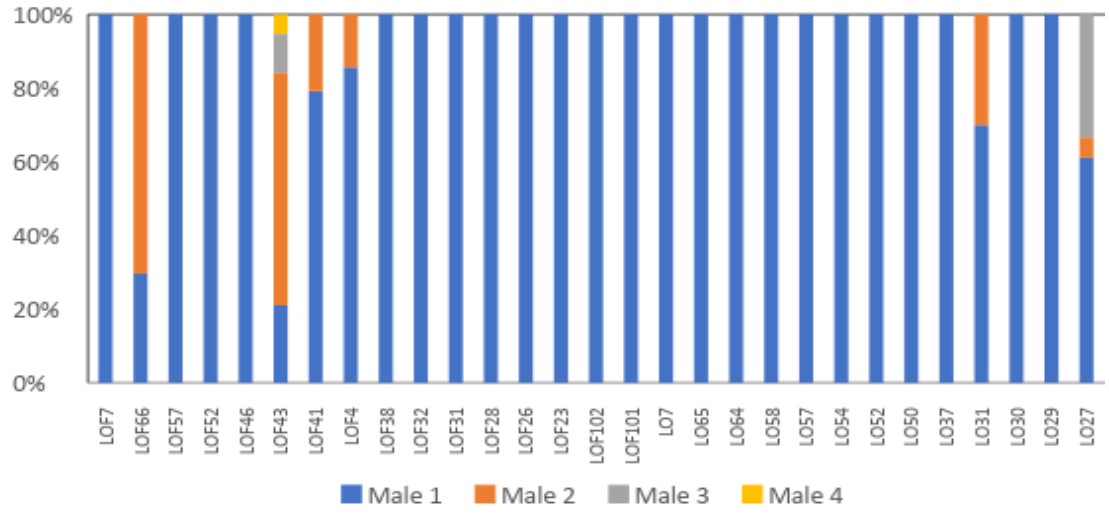

Figure 2. Percentage of male genotype contribution to nests of individual olive ridley turtles (*Lepidochelys olivacea*) in São Tomé island. Analogous colours differentiate the different males fathering offsprings in each nest. There are no shared males between nests of different females.

Sex-biased dispersal

Mean relatedness was significantly higher for females than amongst males (1596 and 561 female and male dyads analysed, respectively; females, $r = -0.010$, male $r = 0.005$; $z = 1.959$, $p = 0.025$), suggesting that there is male-biased dispersal. Results obtained from the Assignment Index (AI_c) tests indicate negative and lower AI_c values for males (mean = -0.448 ± 0.385, n = 18) than for females (mean = 0.139 ± 0.220, n = 58), but differences were not statistically significant ($p = 0.749$).

DISCUSSION

Olive ridleys use highly productive pelagic and oceanic areas to feed, a nomadic behavior that contrasts with most species of sea turtles, which establish foraging territories in coastal waters, and exhibit high levels of philopatry. The general absence of population structure within ocean basins is particularly obvious for the Atlantic, where all olive ridley populations, including the one here studied, are fixed to one haplotype (F), with the only known exception being the Surinam population (two haplotypes, E and F) (Bowen et al. 1997). In such populations, mating behaviour and male-biased dispersal play an important role promoting genetic and demographic connectivity, which ultimately defines the spatial scale of their management in nature (Avise, 2004; Waples & Gaggiotti, 2006). Our study shows evidence of polyandry in the São Tomé population, with the incidence of multiple paternity falling within the range of frequencies observed for other populations around the globe, from 75% and above (Jensen et al. 2006; Duran et al. 2014) to levels similar to that observed in this study (20-30%, Hoeckert et al. 2002; Jensen et al. 2006).

The frequency of polyandry in sea turtles, as inferred by the multiple paternity rates, is now known to be considerably variable both intra- and interspecifically, as well as geographically, likely in response to factors such as mate availability or inbreeding risk (recently reviewed by Lee et al. 2018; see also Bowen & Karl, 2007; Tedeschi et al. 2015). Our ability to determine how many males actually contribute to the nesting population in São Tomé is limited by the temporal scope of our sampling and the accurate assessment of skew in long-lived species like marine turtles requires assessing paternity across years. A stronger sampling regime would also allow, for instance, information of male remigration intervals and sperm storage within and between years, as well as it would extend the ability to apply capture-mark-recapture-type analyses for estimation of the number of males. However, the paternal assessment allowed us to calculate the observed operational sex-ratio (OSR) for the studied interval which should be proportional to the number of males at the breeding area before the nesting season (Hays et al. 2010; Stewart & Dutton, 2011). We observed a relatively balanced OSR, with a slight skew towards the males, a result which corroborates the observed frequency of multiple paternity for this population, as the spatial and temporal availability of males have been linked to polyandric behaviour (Birkhead & Pizzari, 2002; Jensen et al. 2006). The OSR is the key determinant of population viability, as it indicates the proportion of males to females that are ready to mate at any one time (Berglund, 1994; Kyarnemo & Ahnesio, 1996; Weir et al. 2011), and it ultimately reflects the underlying genetic variation of the population. Polyandry is a known strategy to avoid inbreeding and promote sperm competition (Treguenza & Weddel, 2002; Bretman et al.

2009), and indeed inbreeding was not detected in the São Tomé population, as overall F_{is} values for this rookery do not deviate significantly from zero. Male reproductive skew is a key parameter influencing effective population size (N_e), with N_e being larger the more evenly reproduction is distributed amongst males within the population (Hartl, 1988). However, the estimated effective population size (N_e = 55.2) is much lower than the N_e = 500 proposed by Lynch & Lande (1998) as the minimum needed to maintain equilibrium between loss of adaptative genetic variation due to genetic drift and its replacement by mutation.

It appears that moderate levels of male-mediated gene flow in this population, as evidenced by the relatively high genetic diversity at the nuclear level compared to the lack of genetic diversity at $_{mt}$DNA and the lower relatedness observed within this group as compared with the females, may be at insufficient levels to prevent the loss of genetic diversity that will probably result from the severe population bottleneck detected for this population. The results obtained in this study must be therefore interpreted with caution. It is plausible to suggest that the slight male skew observed in the operational sex ratio of olive ridley turtles in São Tomé could simply be a reflection of the directed harvest of females or of unintentional mortality that is focused near the nesting beach. Another concern is the dispersal ability of this species in the Gulf of Guinea region; studies assessing dispersal of other sea turtles nesting in São Tomé and Principe using genetic markers all point towards genetic differentiation of the local rookeries (Formia el al., 2006; Monzon-Argüello et al. 2010; Hancock et al. *in press*) and to very limited dispersal, highlighting the vulnerability of the local rookeries to exploitation. Although none of the studies assessed the environmental and ecological mechanisms that lead to the isolation of these rookeries, and olive ridley turtles typically follow distinct life patterns from other species which are likely to result in distinct patterns, interpretations of the extent of gene flow between São Tomé and several larger nearby rookeries (e.g. Bioko island and Gabon) remains limited due to incomplete assessment of microsatellite diversity of this species in the region. The São Tomé rookery, representing a relatively small population, could potentially be an important source of genetic diversity in the region; its relative role as either a sink or source of genetic diversity must be assessed by studying the current boundaries and level of geneflow between the different rookeries.

CONCLUSION

The prevalence of polyandry in this population, and evidence of male-mediated gene flow, likely to promote genetic exchange between São Tomé and the nearby rookeries of Gabon and Bioko contradict our findings of a bottleneck for this population and of a low effective population size, thus suggesting that this population may be more isolated than expected. Further sampling of the larger rookeries of other Gulf of Guinea islands and of the mainland, and complete assessment of geneflow at a regional level could resolve potential sub-structuring of this species in the region and are highly recommended. These observations strongly suggest high vulnerability to exploitation of the adults, particularly females, for human consumption on São Tomé island, and taking these into account, we alert to the need of careful management and enhance protection of adult females of this rookery to avoid local collapse.

Acknowledgements

We would like to acknowledge in Programa Tatô team in São Tomé Island, in particular Martinho Alves Rosa and his team in Micoló; Hipólito Lima's sons and others working in Morro Peixe and M. Branco, who collected most of the samples often under challenging conditions; S. Lopes, P. Ribeiro and S. Silva (CTM/CIBIO). Permits were provided by National Agency for the Environment (DGA) and Eng. Arlindo de Carvalho. This study was funded by national funds through FCT - Fundação para a Ciência e a Tecnologia by a doctoral scholarship awarded to JMH (PD/BD/52599/2014). HG is supported by a postdoctoral Grant from FCT (SFRH/BPD/102966/2014), and FS is supported by FEDER (COMPETE, POCI-01-0145-FEDER-006821) and FCT (UID/BIA/50027/2013) funds. All samples used in this study were collected and exported under the CITES permits 15ST00009/AC; 17ST000001/AC.

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CHAPTER 4

TARTARUGA SADA

HAWKSBILL TURTLE

Eretmochelys imbricata

PAPER 5

Reproductive biology and conservation status of the critically endangered Hawksbill sea turtle on its main rookery in the Eastern Atlantic

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"Most people are heartless about turtles because a turtle's heart will beat for hours after he has been cut up and butchered. But the old man thought, I have such a heart too".

– Ernest Hemingway

In preparation

Reproductive biology and conservation status of the critically endangered Hawksbill sea turtle on its main rookery in the Eastern Atlantic

ABSTRACT

The hawksbill sea turtle (*Eretmochelys imbricata*) is a critically endangered species, and the nesting population in the Eastern Atlantic is considered one of the most threatened sea turtle populations in the world due to its low numbers and genetic isolation from other rookeries. We conducted the first detailed study of the nesting biology and ecology of *E. imbricata,* on its main rookery in the Eastern Atlantic, the São Tomé and Príncipe islands. Reproduction was monitored for the first time in all known and potential beaches of the archipelago during two consecutive nesting seasons (2015-16 and 2016-17). Analysis of nesting distribution patterns and nesting abundances indicated that the nesting peak occurs in December and January, with nesting densities varying between beaches and islands. We identified a total of 39 unique sites in São Tomé and 19 in Príncipe that host hawksbill reproductive activity. The major nesting area for the hawksbill in São Tomé in Príncipe is located in Rolas islet (off São Tomé), which represents 71% of all activity in São Tomé island, and 52.8% of all activity in the archipelago. Curved Carapace length and clutch size were smaller than other rookeries in the Atlantic. This information facilitates the designation of index nesting beaches for long-term monitoring in the archipelago and allows targeted monitoring and protection efforts, both at a spatial and temporal level, thus reducing field effort. We found that a significant proportion of hawksbill nesting occurs in well protected beaches, but with high human impact.

Keywords: *Eretmochelys imbricata*; Eastern Atlantic; Gulf of Guinea; spatial analysis

INTRODUCTION

The hawksbill turtle, *Eretmochelys imbricata* (Linnaeus, 1766) is a moderate-sized sea turtle reaching a maximum carapace length (CL) of 90 cm (Van Dam & Diez, 1998; Musick & Chaloupka, 2017) and a body mass up to 112 kg (Santos et al. 2010), circumtropically distributed in coastal waters, where it inhabits rocky coastlines, coral reefs, estuaries, and lagoons with mud substrates (Bjorndal and Bolten, 2010; Gaos et al. 2011) of at least 108 countries of tropical and sub-tropical Atlantic, Indian and Pacific Oceans (Groombridge & Luxmoore, 1989; Mortimer & Donnelly, 2010 and references therein). The species is known to nest in at least 70 countries, with most nesting occurring at low density (Groombridge & Luxmoore, 1989). In the Eastern Atlantic Ocean Hawksbills are known to nest along the West coast of Africa, although its status and distribution are poorly known, but the most significant nesting is thought to occur in Guinea Bissau, where 200 females may nest annually (Catry et al. 2009), Bioko (Tomás et al. 2010) and São Tomé, and Príncipe (Graff, 1996; Monzón-Arguello, 2011), with sporadic nesting also occurring in the Cape Verde Islands, Mauritania, and Senegal (Fretey, 1998). Although the spatial distribution of hawksbill genetic stocks within West Africa is still unclear due to incomplete sampling, mixed-stock analysis of juvenile and adult hawksbill turtle populations, coupled with a recent study of the genetic composition of the São Tomé and Príncipe rookery suggest migratory connectivity between foraging and nesting aggregations of East and West Atlantic regions (Monzón-Arguello et al. 2010; Proietti et al. 2014) and high genetic isolation of the Eastern Atlantic stock, which diverged from the Indo-Pacific phylogenetic clade and shows low genetic variability (Monzón-Argüello et al. 2011).

A review of the global status of hawksbills authored by Groombridge & Luxmoore (1989) concluded that hawksbill populations were depleted or declining in 56 of the 65 geopolitical units for which some information on nesting density was available, with declines well substantiated in 18 of these areas. The species was included in the Appendices of CITES in 1975 (Atlantic population in Appendix I) and listed in Appendix I and Appendix II of the Convention on Migratory Species (CMS). Recent population estimates indicate that in Central Africa less than 100 hawksbills nest per year (Mortimer & Donnelly, 2010), and presume that the islands of São Tomé and Príncipe harbour one of the last remaining hawksbill nesting aggregations in the region. These findings have led to some authors to propose this as one of the most threatened Regional Management Units for Marine Turtles (RMU's; Wallace et al. 2010). In a survey conducted in São Tomé and Príncipe by Castroviejo et al. (1994), the hawksbill population was described as being severely depleted due to overexploitation for the

tortoiseshell trade, an activity that was recorded in these islands as early as the late 1800s, and has targeted hawksbill of all sizes due to their high value for craftwork (Keinath & Musick, 1991; Ferreira, 2015) until new legislation come into force in July 2014 (Law Decree n. 8/2014). This law implements a complete ban on the harvesting of sea turtles and trade of their by-products, including tortoiseshell, meat and eggs.

Because estimates of an average age at maturity are at least 20 years for hawksbill sea turtles (Diez & Van Dam, 2002; Snover et al. 2013), long-term monitoring is essential to document true population change, but even more important is to obtain baseline estimates of population size and reproductive output, which have not been established for the East Africa population. Population size estimates are hindered by limited access to reproductive males and to all non-reproductive segments of the population, as the most commonly used method of monitoring population trends is to count the number of females arriving annually at nesting beaches (Board, 2010). Data on the number and characteristics of the nests themselves – including clutch sizes and biotic and abiotic factors affecting hatching success - can be collected without observing the female, as tracks and nests alone can be monitored to provide accurate estimates of nesting patterns, particularly at sites that cannot be monitored regularly. Because estimates of population changes over time will eventually be determined by the numbers of nesting females or numbers of nests deposited (Bjorndal et al. 1999; Balazs & Chaloupka, 2004; de Pádua Almeida et al. 2011), the collection of these baseline data is critical to detect trends over time or conduct population viability analyses, key requirements of any conservation program (Beissinger & Westphal, 1998; Hays, 2000; Heppel et al. 2002).

Sea turtle nesting monitoring started in 2002 in São Tomé by the NGO Marapa, and in 2012 in Príncipe by the NGO Associação Tartarugas Marinhas, later replaced by Programa Têtuaga. Seasonal nesting estimates have been based in monitoring of areas from where higher levels of nesting of the two main sea turtle species occurring in the country (olive ridley and green sea turtles) were known, and that additionally were easily accessed (i.e. proximity to a community, road access). Because financial, political and logistical difficulties frequently limit the extent of the area surveyed, in the seasons of 2015-2016 and 2016-2017 a comprehensive survey of all known and potential hawksbill sea turtle nesting sites was conducted in both islands to identify cryptic nesting sites, since this species is known to be exhibit high levels of philopatry and often nests in small, secluded beaches. Ultimately, the goal was to ensure that no important nesting areas were overseen or unmonitored in future efforts, and that the selection of sites for protection and monitoring included beaches of high conservation interest. A final goal was to evaluate to what extent existing protection efforts included important hawksbill nesting habitat.

This is the first comprehensive survey to cover all potential nesting sites in São Tomé and Príncipe islands, including beaches outside the regularly monitored areas. Data on temporal and spatial distribution of this species are complemented with biometric and reproductive output data. Additionally, we incorporate information on major threats and on the relative vulnerability of each beach where this species is known to nest. Ultimately, our goals are to provide baseline estimates for future trend analysis and to provide recommendations on priority intervention areas for the largest remaining hawksbill sea turtle rookery in the Eastern Atlantic.

METHODS

Study Location

São Tomé and Príncipe islands lie in the Gulf of Guinea, approximately 250 km west of Gabon; climate is equatorial with an average daily temperature of 27 ºC, and high rainfall which increases considerably along the north-south gradient, with one major rainy season that runs from October to May. With areas of 854 km^2 and 142 km^2 respectively, São Tomé and Príncipe islands comprise together over 269 km of coastline (Burke et al, 2001), and contain 102 (São Tomé) and 35 (Príncipe) beaches respectively with lengths ranging between 0.035 to 1.5 km in length.

Nesting activity monitoring protocol

A total of 29.1 km of coastline is potential or known sea turtle nesting sites in São Tomé, and 12 km in Príncipe. In São Tomé 24.5 km are monitored on a daily basis while in Príncipe all beaches are monitored from early October through late March each year, the period coinciding with the nesting season of the four species that nest regularly on this archipelago: green (*Chelonia mydas*), olive ridley (*Lepidochelys olivacea*), leatherback (*Dermochelys corieacea*), and the hawksbill (*Eretmochelys imbricata*). Hatching typically begins in December and lasts until May. Because these islands have numerous non-contiguous nesting beaches, it is impossible to distribute monitoring resources to all sites to ensure maximum coverage. Although regular sea turtle monitoring was initiated in São Tomé in 2003, comprehensive island surveys in both islands have only been conducted since 2015. The data analyzed in this study corresponds uniquely to the 2015-16 and 2016-17 reproductive seasons, and were used to characterize spatial and temporal distribution of the nesting activity and to estimate the number of nesting females.

The comprehensive surveys followed two distinct monitoring schemes: 1) full monitoring of known index beaches in each island (Scheme 1), and 2) diffuse coverage across all sites where nesting was not known (Scheme 2), following recommendations by Board (2011) for situations where numerous sites are used by the same population. Scheme 1 assumes that annual abundance patterns observed via comprehensive monitoring of index beaches (selected because they host a significant proportion of the overall nesting population within a region or other defined unit) reflect a broader pattern that occurs at all other beaches used by the same nesting population. In cases where several, dispersed sites host nesting, but none at significantly high levels to be proposed as index beaches, this approach might not be appropriate (Girondot et al. 2007). In such cases, a more favorable protocol (Scheme 2) would consist of monitoring many sites at low levels of survey effort, and then analyzing abundance estimates across sites (diffuse coverage) (see Delcroix et al. 2014).

On beaches monitored under Scheme 1, effort was standardized to take place each night between 6 p.m. and 1 a.m., by teams of 2 trained field technicians, each covering 1.5 km stretch of contiguous coastline; morning surveys were scheduled at a daily basis to record any activity that was missed by the teams leading the night patrol, and to ensure a complete record of all nesting activity for each beach each night. Scheme 2 included early morning surveys 1-3 times per week during the two month period corresponding to the peak nesting season (estimated to occur between December and January each year) and was applied to all suitable beaches with unknown, uncertain or historical nesting activity (Fig. 1a,b). All detected tracks, in either monitoring scheme, were crossed over using a clear zigzag pattern to avoid repeating observations in subsequent surveys.

Data collection on reproductive parameters and nesting females

During night patrols, the teams of local field technicians collected biological data on nesting females encountered, including biometric data, and proceeded to tag or collect tagging data for individual identification. Tagging was done placing a pair of Inconel flipper tags (National Band and Tag Co., Style 681) on the trailing edge of each of the fore-flippers after egg laying. Biometric data collected includes the minimum curved carapace length (CCL) and width (CCW) recorded to ± 0.1 cm, and was obtained using a flexible measuring tape, as described in Bolten (1999). Due to low observation rates on Príncipe (< 5 females), we only analyzed data from São Tomé for this study.

In São Tomé island, clutches laid on index beaches were primarily relocated to the enclosed hatchery located on Inhame beach immediately after laying or soon after (if the turtle was intercepted or the nest was found during the night patrol), or in the early hours of the morning during morning survey; to date relocations are not conducted on Príncipe island. Eggs are counted and placed in plastic buckets (5 l) and then reburied in hatchery sand using the same dimensions of the original nest. Clutch frequency (i.e. number of observed clutches for an individual female throughout the season) was not calculated as this parameter is dependent upon survey intensity and may be inaccurate due to variation among females. Internesting interval was estimated as the number of days between a successful nesting of a tagged female and the subsequent nesting attempt by the same turtle (Alvarado & Murphy, 1999). Because calculating the internesting interval requires a representative sample of turtles nesting at least twice during the reproductive season (N of 100 or more is recommended, Murphy, 1999), we provide only indicative values.

Modelling of temporal and spatial distribution of nesting activity

The nesting season model assumed an 8 months long season (September 1st – April 30th), following the pattern observed elsewhere: a low number of nests at the start and end of the season, a nesting peak in the middle of the season, and sporadic nesting events outside the nesting season. Inter and intra-seasonal modelling of nesting spatial and temporal activity was performed with the R package "phenology" available in the Comprehensive R Archive Network (Girondot, 2018), a parametric, asymmetric sinusoidal model that essentially fits a curve to sea turtle nesting data to generate estimated values from missing monitoring days (i.e. when no count is obtained due to no monitoring), thereby generating an estimate of the total number of nests for the duration of a season. This model also produces confidence intervals to allow for evaluation of the uncertainty associated with the total abundance estimates. The information about the shape of this curve was obtained from the sum of all nesting activities recorded on the beaches monitored under Scheme 1, and from modelled values for beaches monitored under Scheme 2, resulting in a complete estimate of seasonal abundance (Delcroix et al. 2014). The model of nesting seasonality is based on Girondot (2010; 2018). This model was preferred among the several available because: (i) it performed among the best based on an extensive test (Whiting et al. 2014), (ii) its parametric definition allows the standard error to be minimized and (iii) the parameters have direct biological interpretations.

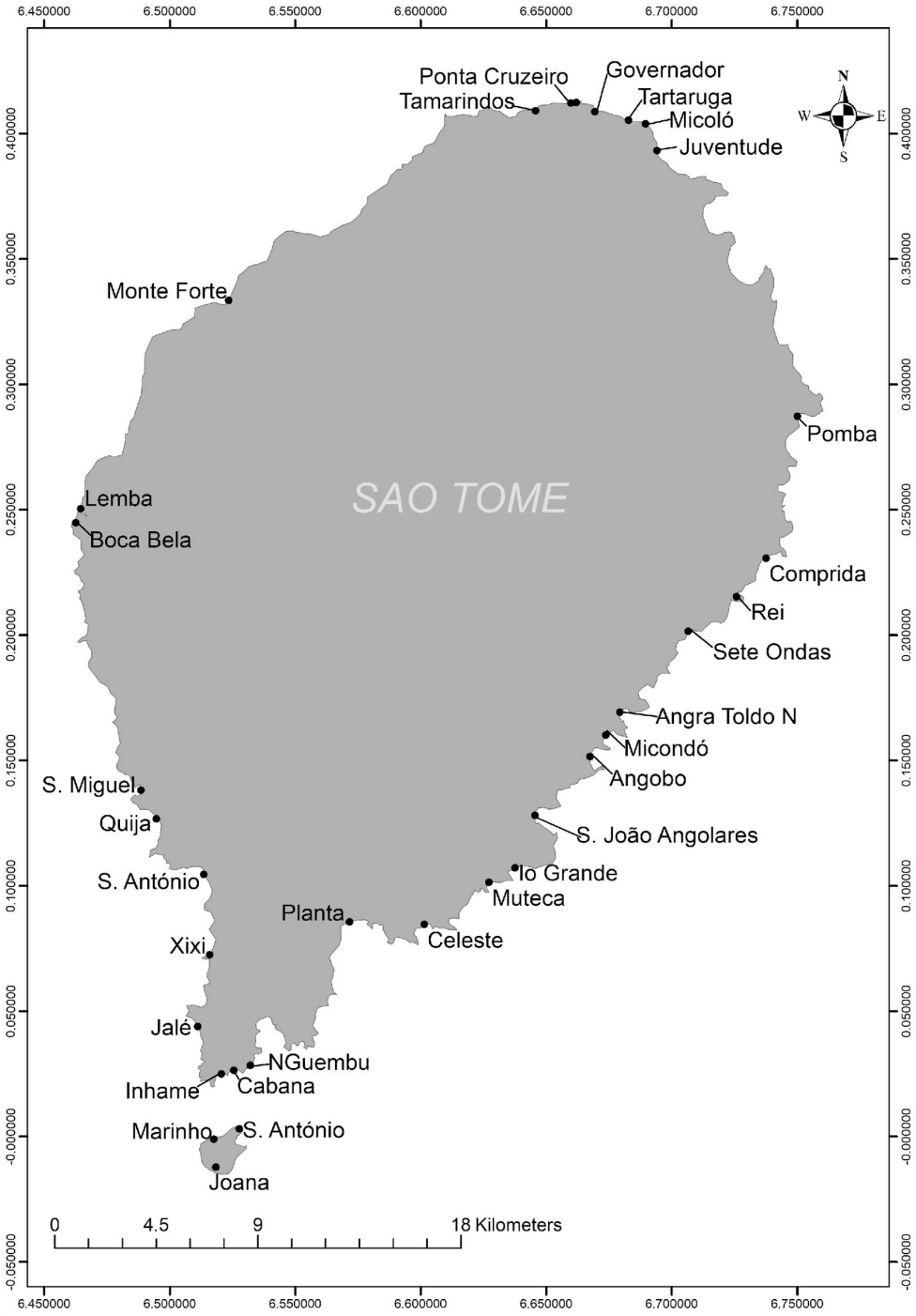

Figure 1a. Map of São Tomé island, and location of the beaches where monitoring was conducted during 2015-2016 and 2016-2017 seasons by Programa Tatô.

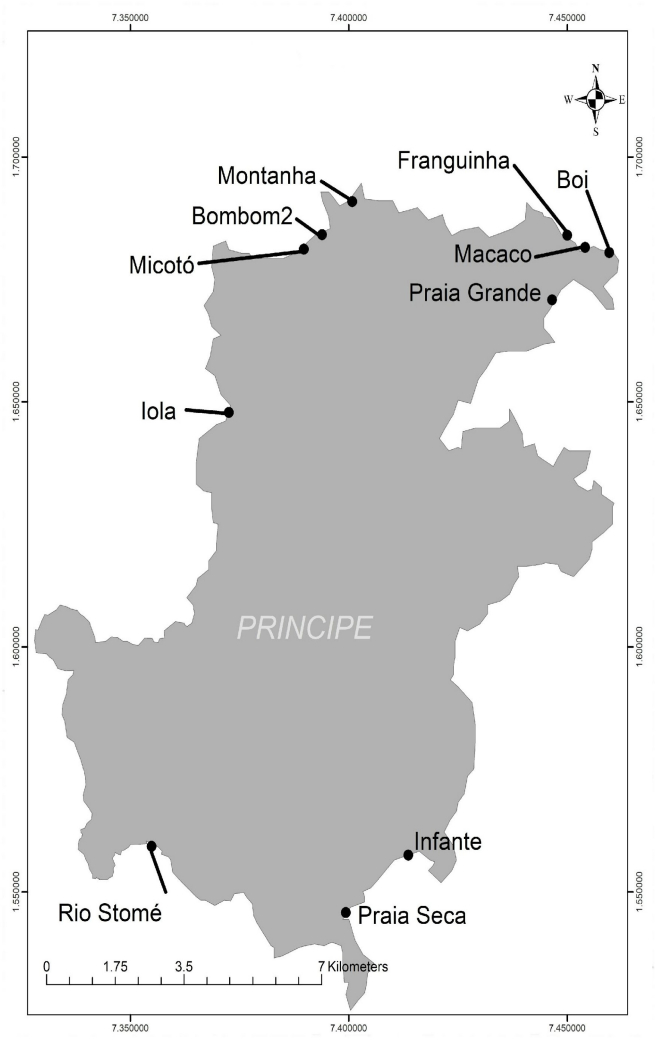

Figure 1b. Map of Príncipe island, and location of the beaches where monitoring was conducted during 2015-2016 and 2016-2017 seasons by Programa Têtuaga.

Threat exposure

A rapid assessment of potential human impact was conducted for each nesting beach for which hawksbill nesting was observed in São Tomé and Príncipe islands (39 and 21 beaches, respectively), using the "Nesting Beach Indicator" version 1.1 (developed by Cousins et al. 2017). This screening tool, meant to be indicative, rather than conclusive, includes a predictor of human impact on the beach, indicating impact factors scores that range between 1 and 5 (1 = lowest, 5 = highest score), taking into consideration the existence of fixed or semi-fixed structures behind the beach, potential obstructions to nesting females, levels of disturbance and evidence of light pollution exposure. We further categorized the level of human impact as "High" (human impact is likely to deter nesting), "Medium" (human impact may affect nesting) or "Low" (human impact unlikely to affect nesting. Protection regimes were categorized as

"High" for beaches where active protection of nesting females was enforced during night-time patrols, "Moderate" for those beaches where there was no active protection of nesting females, but regular presence of project staff on the beach could potentially deter human impact (e.g. female and nest harvesting) throughout the season and "None" for the remaining beaches.

RESULTS

Reproductive parameters

During the study period a total of 76 individual hawksbills were identified through flipper tagging in the island of São Tomé. The mean CCL was 79.7 ± 6.1 cm (range 68 - 93), and the CCW was 71.2 ± 5.38 cm (range 60 - 84). The reproductive output was 125 ± 28.9 eggs per clutch (n = 145). Recapture rates of females in renesting events were very low (12 %, n = 10), but allowed the calculus of the internesting period of 18 days (modal value; range 12 – 20 days, n = 10). Two females were recorded nesting in two consecutive seasons. A total of 42 clutches were relocated to the hatchery at Inhame beach. The mean hatching success from these nests was 71.1 ± 18.8 % (mean ± SD; range 41.1 - 100) and mean emergence success was 63.7 ± 22.67 % (range 28.4 - 100). The incubation period to emergence in the hatchery was 61.8 ± 6.3 days (range 50 - 82).

Temporal and spatial distribution of nesting activity

The estimated number of Hawksbill tracks in São Tomé and Príncipe was 314 and 445 in 2015-16 and 2016-17 season respectively (Fig. 2). The proportion of tracks with a nest was estimated as 0.17 with 95% confidence interval being from 0.12 to 0.23, resulting on an estimated range of 37.7 – 72.3 nests in 2015-16 and 75.6 – 102.3 in 2016-17. Based on range of estimates of the number of nests for each season, and considering an average clutch frequency of 3 nests for this species, we estimate a minimum of 13 – 25 and a maximum of 25 – 34 individual females nesting in 2015-2016 and 2016-2017 seasons respectively in the whole archipelago.

We identified a total of 39 unique sites in São Tomé and 19 in Príncipe that host hawksbill reproductive activity. Table S1 presents the estimated total number of nesting activities on the major groupings of beaches on each of the two islands in each season; the distribution map is represented in Fig. 3. The major nesting area for the hawksbill in the country is located in Rolas islet, which represent 71 % of all activity in São Tomé island, and 52.8 % of all activity in the archipelago, particularly in Joana and Marinho, where we estimated a combined nesting activity

of 225 ± 38.2 tracks during the 2016-2017 season. Minor nesting sites in the archipelago include the beaches of Inhame, Cabana and Jalé (7% combined), Celeste (4.3%), Grija (2.3%) and Io Grande (1.4%), in São Tomé and the stretch of coast between Bom Bom and Ponta Margarida (8.1%), Praia Grande (2.6%), Cemitério (2.5%), Praia Seca (1.7%) and Infante (1.2%) in Príncipe.

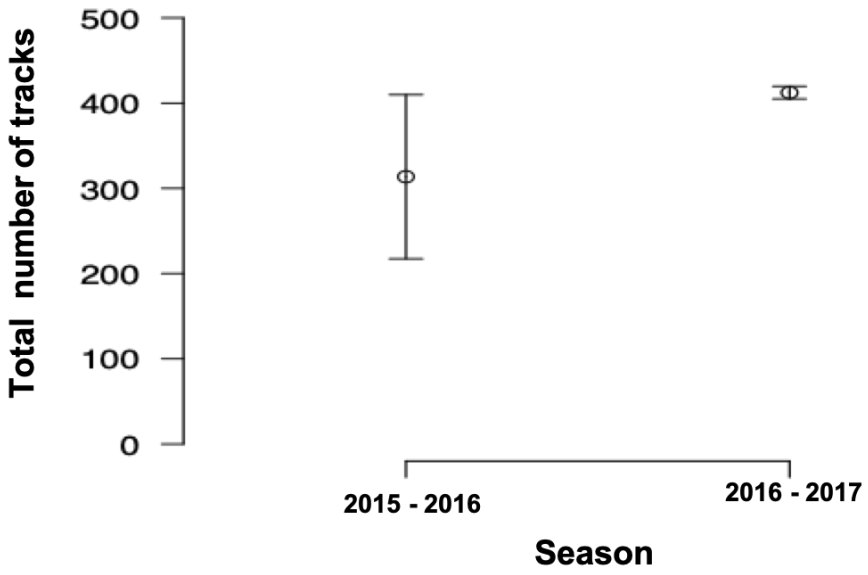

Figure 2. Estimated total hawksbill sea turtle (*Eretmochelys imbricata*) tracks in São Tomé and Príncipe during the two seasons studied and associated 95% confidence intervals. Small confidence intervals in 2016-2017 likely reflect increased monitoring effort in Rolas islet during that season, reducing error in estimates.

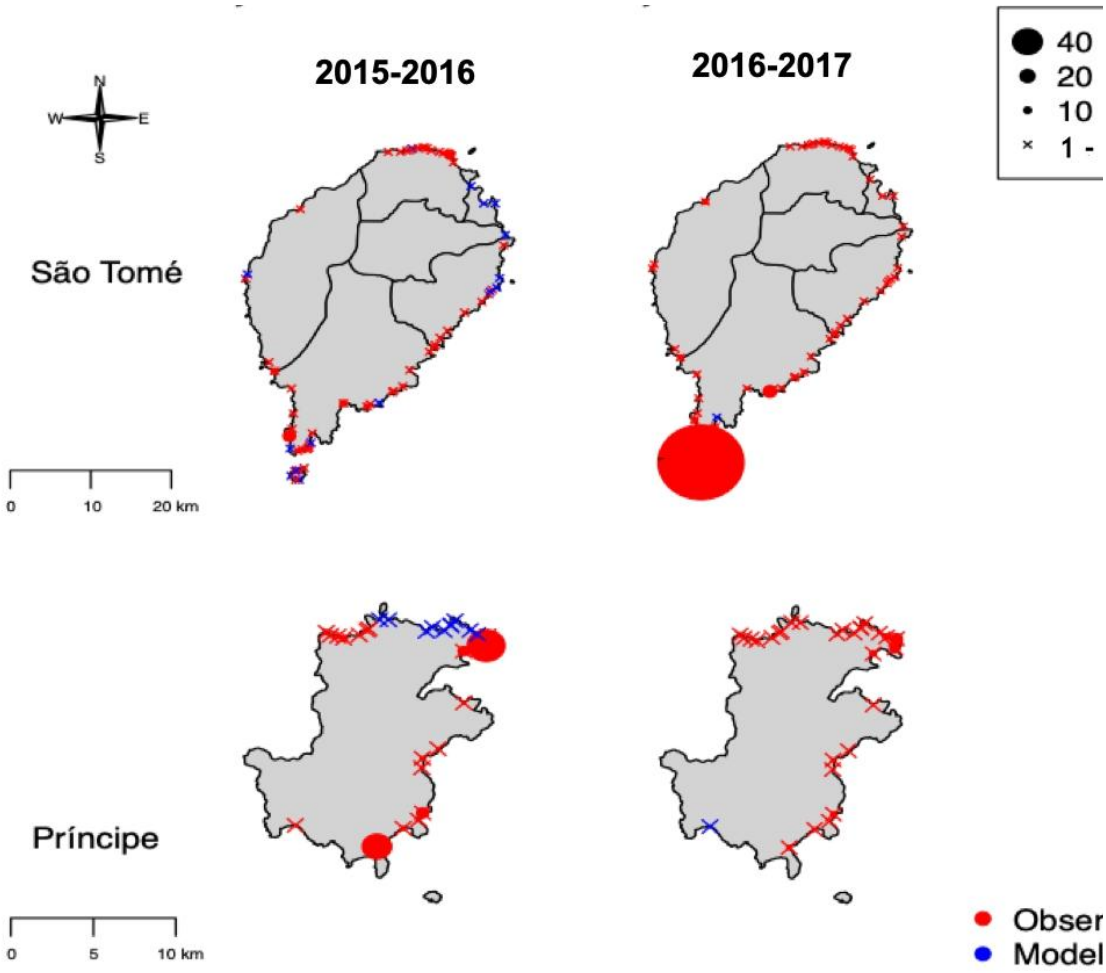

Figure 3. Spatial distribution of the nesting activity in São Tomé and Príncipe islands. Rolas islet monitoring changed from Scheme 2 to Scheme 1 between the two seasons studied, explaining the higher abundance of nesting activity in 2016 – 2017. Key: observations (**red**), modelled frequencies (**blue**) for sites where no nesting was observed, but was likely to occur.

Nesting activity of hawksbill turtles is highly concentrated on the southern shore of São Tomé island, with only 1.9% occurring between the northermost beaches of Lembá and Micoló. In Príncipe, nesting is more diffused, with most activity recorded in both north and southern beaches (Fig. 4).

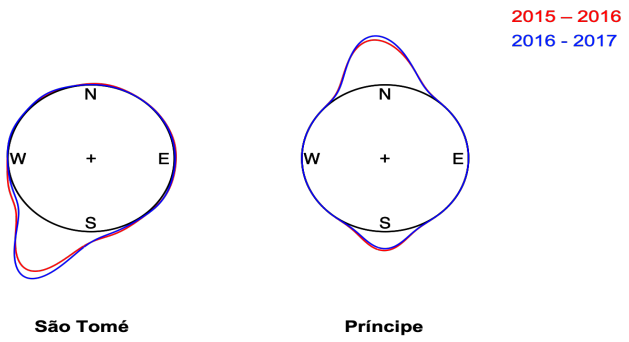

Figure 4. Distribution patterns of hawkbsill turtle (*Eretmochelys imbricata*) nesting activity in São Tomé and Príncipe islands, as modelled by the R package *phenology*, showing clear preferences for the southern shores in São Tomé and northern and southern shores in Príncipe.

Analysis of temporal distribution of the nesting season shows that it starts at the end of October, and extends to late March, with nesting activity peaking between late December and early January (Fig. 5).

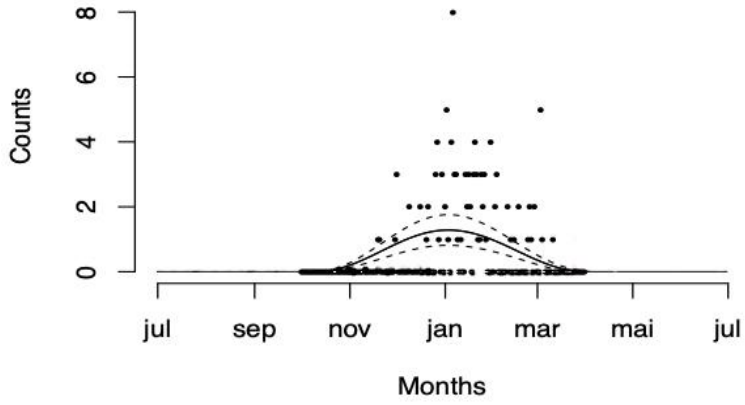

Figure 5. Hawksbill temporal nesting distribution during the 2016-2017 season in Joana beach, Rolas islet. The central curve is the best fitted model and the upper and lower curves are the error envelopes (± 2 s.d.).

Threat analysis

In terms of human impact factor, hawksbill nesting is thought to already be or likely to be affected in the future by human impact in a total of 30 beaches in São Tomé island where nesting has been confirmed. Highest impact factor (human impact likely to have deterred significant nesting activity) was assigned to 16 of the monitored beaches, all of which are located near coastal communities, resorts, or used for livestock rearing (cows or pigs) or sand-mining. Therefore, 75% of the beaches used by hawksbills in São Tomé island are currently under human pressure (Figs. 6; 7; see Table S2 for full assessment). Of the 18 sites with > 5 activities recorded per season, 16 are currently fully protected and monitored under Scheme 1 (n = 6 in São Tomé, n = 10 in Príncipe).

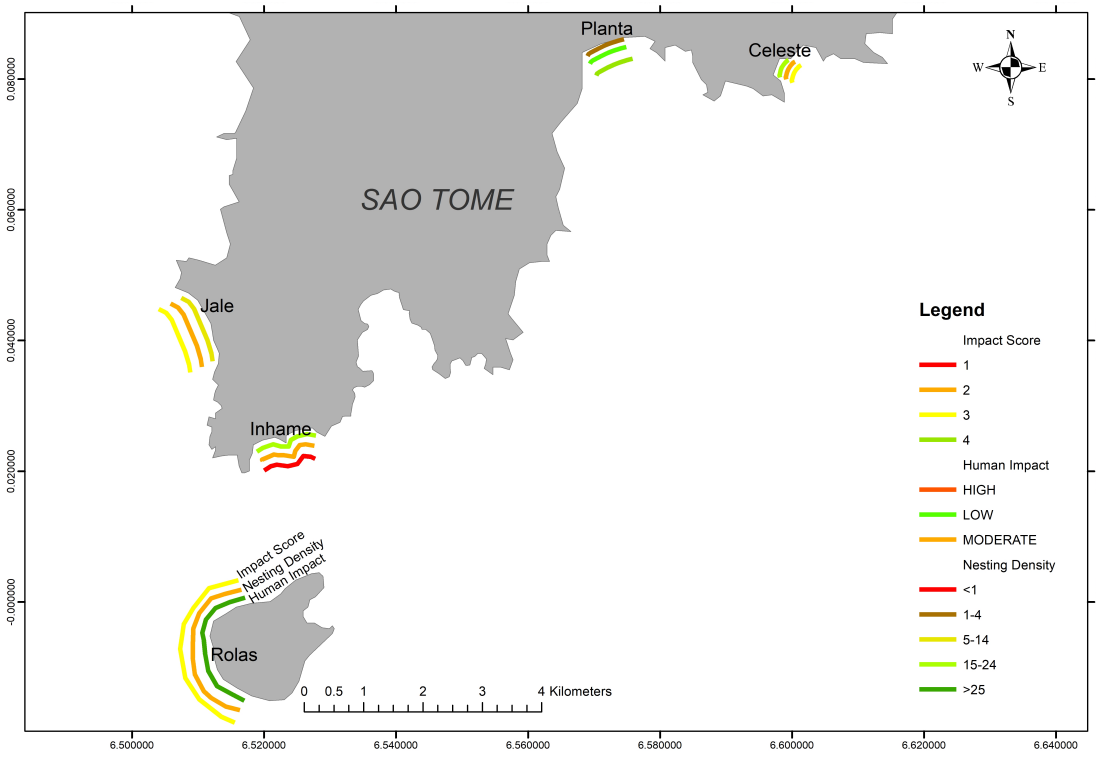

Figure 6. Threat analysis of the key nesting sites for the hawksbill sea turtle (*Eretmochelys imbricata* on São Tomé island. See methods for description of categories used.

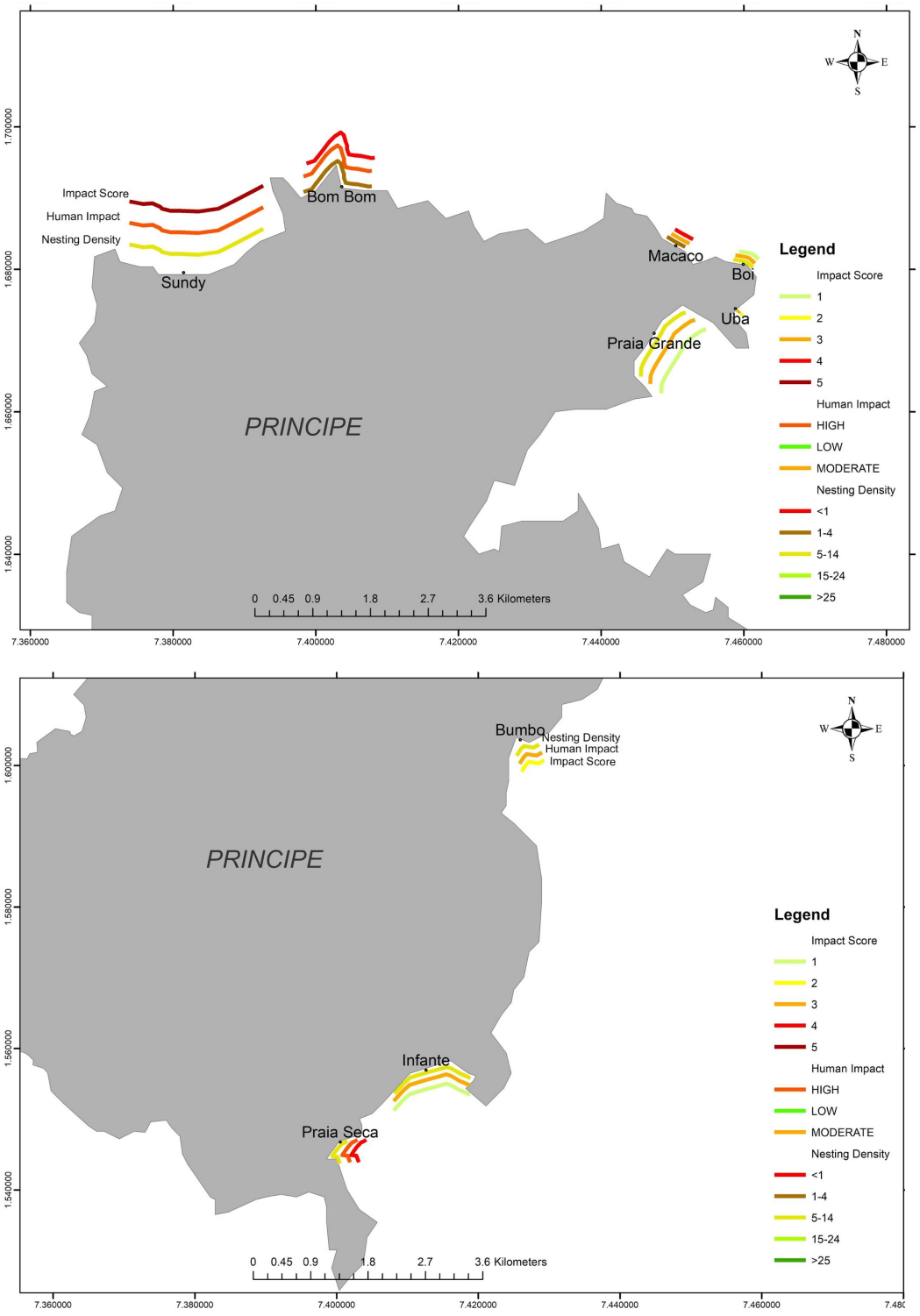

Figure 7. Threat analysis of the key nesting sites for the hawksbill sea turtle (*Eretmochelys imbricata* on Príncipe island. See methods for description of categories used.

DISCUSSION

Reproductive parameters and population status

As genetic studies point to a single hawksbill management unit in the Eastern Atlantic region (Monzón-Arguello, 2011), it is clear that data on the São Tomé and Príncipe populations is critical for developing a robust demographic picture of this management unit. The average curved carapace length (CCL) reported (79.8 cm) is much lower in São Tomé and Príncipe when compared to other populations in the Atlantic, where reported values ranges from 87.6 – 97.4 cm CCL (Barbados: 89.6 cm, Krueger et al. 2011.; Costa Rica: 88.8 cm, Bjorndal et al. 1985 with highest values reported for the Brazilian and Mexican rookeries at 97.4 and 99.4 cm (Marcovaldi et al. 1999, Garduno-Andrade, 1999). This finding could reflect selective harvesting of females on the main nesting beaches for tortoiseshell. São Tomé and Príncipe were one of the major sources of tortoiseshell in Africa, an anthropogenic pressure that could have produced a reduction in mean female size by preferentially removing the older, larger females. However, female size is similar to rookeries in the western Indian ocean, such as Seychelles (85.0 cm, Hitchins et al. 2004); Persian Gulf (Oman (71.6 cm, Hesni et al. 2016) and Saudi Arabia (71.2 cm, Pilcher, 1999). This could be explained by the strong genetic link found by Monzón-Arguello et al. (2011) between the Eastern Atlantic haplotypes and those identified in the Indo-Pacific. These authors suggest a possible colonization of the Eastern Atlantic from the Indian ocean, which could explain similarities.

Mark-recapture efforts conducted during the study period resulted only in the observation of renesting of four females out of 52 identified; although the data is sparse, it suggests an inter-nesting interval of 18 days for the hawksbills nesting in São Tomé and Príncipe, a value which is consistent with the range of 10 to 19 days documented for other rookeries (Bjorndal et al. 1985; Pilcher, 1999; Kamel and Delcroix, 2009). The low recapture rate could be a monitoring artifact, as hawksbills often nest during daytime, or nest in small isolated beaches, therefore may re-nest undetected. Improved monitoring of the main nesting sites, some identified during the study, will allow the determination of important parameters such as renesting and internesting interval and clutch frequency in the future. However, this low recapture rate could be indicative of a higher proportion of older females at this rookery, which lack the reproductive ability to renest. This possible demographic imbalance could be the result of a low rate of juvenile recruitment (Hirth, 1971; Rueda, 1992) due to the anthropogenic pressures experienced over the years (e.g., harvest of reproductive females and eggs), and could be shown by a lower reproductive output as well. When compared to other rookeries, the mean clutch size for the São Tomé females (mean clutch size of 125.59 ± 28.43 eggs) is average, but lower than the

values recorded for the Atlantic, which range from 136.4 in Brazil (Marcovaldi & Laurent, 1996) to 163.8 in Guinea Bissau (Catry et al. 2009). However, the smaller size recorded for the nesting females does not corroborate the hypothesis of an unbalance towards old individuals.

Spatial and temporal distribution

Analysis of spatial and temporal patterns of nesting is of critical importance to conservation and management. Nest site selection and nesting success are influenced by a variety of marine and terrestrial factors (e.g. Weishampel et al. 2003). In this study we conducted a comprehensive 2-year survey to identify critical sites for monitoring and conservation of a reduced, yet spatially dispersed, colony by which we assessed the level of nesting of this species in most available nesting sites. Our results show that although many beaches offer suitable conditions for nesting, hawksbills clearly prefer those sites located on the south of São Tomé, which coincidently or not, receive the highest rainfall and are mainly characterized by steeper slopes, coarser sand (generally yellow or white) and high vegetation cover (Hancock, *pers. obs*). In Príncipe most beaches are similar to those just described and receive similar amounts of rainfall, with the notable exception of the Infante beach, located on the south of the island, which is the only black sand beach in Príncipe and is characterized by a very gentle slope.

Our model indicates that the hawksbill turtle nesting season in São Tomé and Príncipe is similar to other species occurring in West Africa, and typically extends from October to March (absolute range: August - May). It is likely that seasonality of nesting and site selection is tied to climatic factors leading to suitable nest construction and incubation environments. There is a marked, although limited, seasonal pattern in air temperatures and little air temperature fluctuation during the reproductive season, with marine turtle nesting and incubation occurring in the warmest months.

Nesting activity is generally low throughout each island, with only few beaches with estimated annual count >15 activities; in most beaches estimates range between 1-4 tracks a year, with the exception of Rolas islet, which is clearly the main nesting site for this species in the archipelago. Modelled detection profiles show that regardless of the study species, and particularly in reduced populations where nesting is widely dispersed, such as the hawksbill, a large proportion of sandy coastline must be surveyed every year in order to detect all nests, raising monitoring challenges. However, our data show that in cases where resources are critically low, it may be possible after an initial multi-year assessment such as ours to focus efforts on known areas of relatively high intensity, which in the case of São Tomé and Príncipe include the southern beaches of Inhame and Rolas islet (all) in São Tomé, and the northern

beaches of Príncipe, particularly Boi, Macaco Uba, Micotó, Ribeira Izé and Margarida, and Praia Seca in the south.

This will facilitate the designation of index nesting beaches for long-term monitoring of population status and allow targeted monitoring and protection efforts, thus reducing field effort. This is equally applicable to the analysis of this population's phenology and estimation of peak nesting activity. In our study the combination of monitoring schemes provided a good overview of temporal and spatial distribution of hawksbill nesting in the archipelago, but it is important to note that curtailing the temporal coverage of beaches to peak nesting months adds additional variance into the monitoring data that further reduce the power to detect trends within the context of profound levels of interannual variation in nesting numbers (Broderick et al. 2001; Bell et al. (2006)) and very low numbers. Moreover, intensive beach monitoring also provides opportunities to carry out surveillance regarding anthropogenic threats such as illegal take.

Analysis of threats and considerations on future outlook

As the nesting densities are so low, our small sample sizes must be given consideration before inferences can be made regarding the spatial distribution of nesting with reference to levels of threat in São Tomé and Príncipe. We found that a significant proportion of hawksbill nesting occurs in well protected beaches, but even there some level of human impact can be scaled down. This is very important, as it is likely that this genetically isolated population may be composed of a very low number of females; for this reason, the loss or addition of one female nesting in one area in each year can significantly impact the population outcome. Harvesting of female turtles was being conducted at an alarming rate, especially in São Tomé island until the implementation of the national decree in 2014, with high rates of annual female mortality for reproductive females, including hawksbill turtles. The local NGOs have been reporting since a steady reduction in female harvesting by humans, largely as a result of continuous education efforts directed at members of the local communities that have produced greater environmental awareness. Efforts to create alternative activities, particularly for turtle sellers and tortoiseshell craftsman have been conducted by Programa Tatô in São Tomé (Vieira et al. 2017), which have been partially successful in reducing the offer of sea turtle products in the capital's market and souvenir stalls. The confirmation of Ilhéu das Rolas as the main nesting site for this species in the archipelago spells positive news as this islet is actively managed by a local resort fully engaged with sustainability practices, and in response to the high nest predation rate by pigs, implemented the use of a turtle hatchery. On the other hand, Príncipe island as a whole was

declared as a Biosphere Reserve in 2012, which resulted in the reinforcement of sea turtle monitoring and protection through the implementation of Programa Têtuaga and its successful "Zero Capture" campaign, which initiated in 2016. Several conservation and research activities are currently taking place in both islands, and a new project aiming at establishing a network of Marine Protected Areas across the archipelago.

CONCLUSION

It is clear that status assessment and ongoing monitoring of the levels of harvest and nesting are essential to inform adequate conservation in São Tomé and Príncipe. Although São Tomé and Príncipe is considered to hold the most important nesting sites in the Eastern Atlantic for *E. imbricata*, because of limited monitoring of nesting females, data are insufficient to establish population trends. Rapid assessments such as those conducted in this study are relatively quick and inexpensive while allowing to gather targeted information, but its scope and sample size are usually limited, like the reliability of the conclusions obtained. However, given the importance of this rookery, the data obtained in this study is a valuable contribution to the knowledge of the reproductive ecology of the hawksbill turtle regionally and globally, and hopefully will help refine ongoing conservation actions.

Acknowledgments

This assessment was a result of intensive field work conducted by staff of Programa Tatô and Têtuaga in São Tomé and Príncipe islands respectively. We wish to thank them as well as other collaborators such as Yaiza Yanes Perez and Marina Branco who conducted many of the daily and surveys in São Tomé island. Francesco Rainieri Lopez, current director of the Pestana Equador resort on Rolas islet was fully supportive of all the activities described, and facilitated important logistic and financial support to monitoring and conservation activities in this islet. The staff working on Inhame and Cabana beaches, as well as the hatchery located in Inhame beach, were funded by Inhame Ecolodge; we thank Sr. Nazaré for his continuous support. This study was funded by national funds through FCT - Fundação para a Ciência e a Tecnologia by a doctoral scholarship awarded to JMH (PD/BD/52599/2014).

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SUPPORTING INFORMATION

Table S1. Location of all beaches monitored in each island and estimated total number of tracks (according to our model)

Beach	Location	Latitude	Longitude	2015-2016		2015-2016	
				N	s.e.	N	s.e.
São Tomé Island							
Joana	South / Rolas	-0.012246	6.518414	139.2	31.1	196.9	38.2
Marinho	South / Rolas	-0.001122	6.517565	20.1	6.1	28.5	8.0
Bateria	South / Rolas	-0.007043	6.513076	2.5	1.6	3.6	2.0
Pomba	South / Rolas	0.000006	6.51893	2.3	1.6	3.2	2.2
Cafe	South / Rolas	0.000399	6.522200	1.9	1.1	2.7	1.5
S. António	South / Rolas	0.003031	6.527673	0.1	0.0	0.1	0.0
Escada	South / Rolas	-0.011932	6.522192	0.0	0.0	0.0	0.0
Cabana	South	0.026444	6.525488	6.7	2.8	9.4	3.8
Inhame	South	0.024954	6.52057	5.5	3.5	7.8	4.8
Piscina	South	0.027969	6.512151	0.1	0.1	0.1	0.1
Jale	South	0.043861	6.511182	9.7	4.0	13.7	5.6
Vainha	South	0.05296	6.514351	0.0	0.0	0.0	0.0
Cova	South	0.029174	6.533215	0.9	0.5	1.2	0.7
Marcacao	South	0.026699	6.530529	0.1	0.1	0.1	0.2
NGuembu	South	0.028341	6.532056	0.1	0.1	0.1	0.1
Cocheira	South	0.032771	6.533425	0.1	0.1	0.1	0.2
Porto Alegre	South	0.035075	6.535234	0.0	0.0	0.0	0.0
Malanza	South	0.047303	6.536625	0.6	0.8	0.9	1.1
StAntonio	South	0.104492	6.513558	1.5	1.3	2.2	1.6
Grija	South	0.126684	6.494663	7.2	3.4	10.2	4.6
Xixi	South	0.072502	6.515956	0.7	0.6	1.0	0.9
SMiguel	South	0.138113	6.488498	0.0	0.0	0.0	0.0
MonteForte	North	0.333438	6.523495	1.4	1.1	2.0	1.5
Lemba	North	0.250392	6.46454	0.8	0.6	1.1	0.8
BocaBela	North	0.244825	6.462562	1.4	1.1	1.9	1.5
Brigada	North	0.411436	6.664800	0.9	0.5	1.3	0.6
Tartaruga	North	0.40534	6.682816	0.0	0.0	0.0	0.0
Guegue	North	0.406619	6.635072	0.0	0.0	0.0	0.0
Micolo	North	0.403853	6.689583	0.0	0.0	0.0	0.1
Juventude	North	0.393249	6.694135	0.0	0.0	0.0	0.0
FernaoDias	North	0.408719	6.669317	0.0	0.0	0.0	0.0
Governador	North	0.412171	6.659735	0.0	0.0	0.0	0.0
Tamarindos	North	0.409101	6.645684	0.0	0.0	0.0	0.0
Caroceiro	North	0.410816	6.647930	0.0	0.1	0.0	0.1
AguaLuge	North	0.401668	6.692099	0.0	0.5	0.0	0.7

Table S.1. (Cont)

AtrasMorro	North	0.408229	6.642245	0.0	0.2	0.0	0.3
Ponte	North	0.340901	6.728060	0.0	0.4	0.0	0.5
SCarlos	North	0.411584	6.654812	0.0	0.4	0.0	0.7
FozRio	North	0.406729	6.678552	0.0	0.0	0.0	0.0
PontaCruzeiro	North	0.41243	6.661926	0.0	0.0	0.0	0.0
Celeste1	East	0.081692	6.598047	13.5	4.9	19.1	6.4
Celeste2	East	0.084568	6.601425	1.0	0.7	1.4	0.9
RibeiraPeixe	East	0.086268	6.611135	0.8	0.5	1.1	0.7
Io Grande	East	0.107141	6.637604	4.4	2.3	6.2	3.3
ColóniaAçoreana	East	0.178303	6.687464	0.9	0.6	1.3	0.9
Planta	East	0.085616	6.571594	2.7	1.5	3.8	2.2
Muteca	East	0.100011	6.626185	2.3	1.0	3.3	1.3
Angra Toldo N	East	0.160145	6.673733	0.7	0.4	1.0	0.7
Angra ToldoS	East	0.156856	6.672396	0.7	0.9	0.9	1.2
Sete Ondas	East	0.201529	6.706574	0.6	0.5	0.8	0.7
Pomba	East	0.287295	6.750049	0.7	0.5	1.0	0.6
Rei	East	0.215332	6.725771	0.1	0.1	0.1	0.2
Micondó	East	0.169257	6.679283	0.1	0.1	0.1	0.2
Abade	East	0.224824	6.733458	0.1	0.1	0.1	0.1
Comprida	East	0.230662	6.737584	0.0	0.0	0.0	0.1
Forma	East	0.228159	6.735074	0.0	0.0	0.0	0.0
Giga	East	0.233337	6.742287	0.0	0.0	0.0	0.0
Milha	East	0.363134	6.713814	0.0	0.0	0.0	0.0
Conchas	East	0.406508	6.621472	0.0	0.0	0.0	0.0
Perigosa	East	0.340767	6.740600	0.0	0.0	0.0	0.1
Messias Alves	East	0.245519	6.745508	0.0	0.0	0.0	0.1
S. João Angolares	East	0.128082	6.645522	0.0	0.0	0.0	0.0
Angobo	East	0.151542	6.667459	0.8	0.7	1.2	1.0
Manuel Jorge	East	0.30058	6.751954	0.0	0.0	0.0	0.0
Príncipe Island							
Uba	North	1.674352	7.458768	18.9	6.3	26.7	8.5
Praia Grande	North	1.670823	7.446521	8.2	4.0	11.6	5.2
Micoto	North	1.681154	7.389707	6.5	3.7	9.2	5.1
Ponta Marmita	North	1.68293	7.371763	5.3	2.7	7.5	3.7
Sundy	North	1.679103	7.381196	5.3	2.5	7.5	3.2
Margarida	North	1.680839	7.373861	2.1	1.0	3.0	1.3
Montanha	North	1.684137	7.393887	2.0	1.0	2.9	1.3
Banana	North	1.690216	7.441794	1.7	0.9	2.4	1.2
Bombom	North	1.690865	7.400796	2.8	1.4	4.0	1.8
Franguinha	North	1.684029	7.450018	1.6	0.7	2.3	1.0

Table S.1. (Cont)

Macaco	North	1.681541	7.454099	1.4	1.0	2.0	1.3
Boi	North	1.680463	7.459617	0.0	0.0	0.0	0.0
Ribeira Izé	North	1.68502	7.395001	1.3	0.7	1.8	0.9
Campanha	North	1.683507	7.426232	0.0	0.0	0.1	0.1
Burra	North	1.684089	7.435764	0.0	0.0	0.0	0.0
Ponta Ramiro	North	1.680326	7.378021	0.0	0.3	0.0	0.5
Cemitério	East	1.567270	7.424220	8.0	3.1	11.3	4.1
Bumbo	East	1.602214	7.424071	4.3	2.4	6.1	3.0
Popa	East	1.68598	7.429540	1.0	0.4	1.4	0.6
Pedrona	East	1.687173	7.439450	0.0	0.0	0.0	0.1
Portinho	East	1.637744	7.446630	0.0	0.0	0.0	0.0
Cabinda	East	1.562787	7.421909	0.0	0.0	0.0	0.0
Praia Seca	South	1.545786	7.399314	5.3	3.3	7.5	4.3
Infante	South	1.557488	7.413629	3.7	2.6	5.2	3.5
Rio S. Tomé	South	1.559279	7.354879	0.9	1.1	1.3	1.4

Table S2. Beach suitability and impact assessment on nesting beaches in São Tomé and Príncipe (based on Cousins et al. 2017), where hawksbill (*Eretmochelys imbricata*) was observed or can occur according to our model (see methods for more details).

Island / Beach	Location	Length (km)	Suitability	NA	IS	HI	LAT	LONG
São Tomé island								
A. Morro	North	0,15	Typical	<1	3	Medium	0,408229	6,642245
Brigada	North	0,40	Typical	1-4	4	Low	0,411436	6,664800
Caroceiro	North	0,35	Typical	<1	4	Low	0,410816	6,647930
Conchas	North	0,30	Typical	<1	2	Medium	0,406508	6,621472
Scarlos	North	0,20	Typical	<1	4	Low	0,411584	6,654812
Agua Luge	North	0,67	Typical	<1	2	Medium	0.401668	6,692099
Lemba	North	0,38	Potential	1-4	2	Medium	0,250392	6,464540
Boca Bela	North	0,40	Typical	1-4	3	Medium	0,244825	6,462562
Monte Forte	North	0,15	Potential	1-4	2	Medium	0,333438	6,523495
Comprida	East	0,30	Typical	<1	4	Medium	0,230662	6,737584
Sete Ondas	East	0,40	Potential	<1	3	Medium	0.201529	6,706574
Angobo	East	0,50	Typical	1-4	3	Medium	0,151542	6,667459
Angra Toldo	East	0,65	Typical	1-4	1	Medium	0,158986	6,673244
Colonia	East	0,70	Potential	1-4	3	Medium	0,178303	6,687464
Io Grande	East	0,55	Typical	5-14	2	Medium	0,107141	6,637604
Micondo	East	0,35	Typical	<1	3	Medium	0,169257	6,679283
Muteca I	East	0,20	Potential	1-4	4	Low	0,100011	6,626185
Planta	East	0,85	Typical	1-4	4	Low	0,085616	6,571594
Celeste	East	0,45	Typical	15-25	3	Medium	0,081692	6,598047
Pomba	East	0,60	Typical	1-4	3	Medium	0,287295	6,750049
Rei	East	0,28	Potential	<1	2	Medium	0,215332	6,725771
Ribeira Peixe	East	0,35	Potential	1-4	1	High	0,086268	6,611135
Cabana	South	0,80	Typical	5-14	3	Medium	0,026444	6,525488°
Cocheira Baixo	South	0,26	Typical	<1	3	Medium	0,032771	6,533425
Cova	South	0,17	Typical	1-4	4	Low	0,029174	6,533215
Quija	South	0,65	Potential	5-14	4	Low	0,126684	6,494663
Guembu	South	0,27	Typical	<1	3	Medium	0,028341	6,532056
Inhame	South	0,50	Typical	5-14	1	High	0,024954	6,520570
Jale	South	1,50	Typical	5-14	3	Medium	0,043861	6,511182
Marcação	South	0,25	Typical	<1	3	Medium	0,026699	6,530529
Piscina	South	<0.05	Potential	<1	4	Low	0,027969	6,512151

Table S2. (cont.)

Santo António	South	1,60	Potential	1-4	4	Low	0,104492	6,513558
Xixi	South	0,33	Typical	1-4	5	Low	0,072502	6,515956
Bateria	Rolas	<0,05	Typical	1-4	5	Low	-0,007043	6,513076
Café	Rolas	0,24	Typical	1-4	1	High	0,000399	6,522200
Escada	Rolas	<0,05	Typical	1-4	5	Low	-0,011932	6,522192
Joana	Rolas	0,18	Typical	>15	3	Medium	-0,012246	6,518414
Marinho	Rolas	0,15	Typical	>15	3	Medium	-0,001122	6,517565
S. Antonio	Rolas	0,90	Typical	<1	2	Medium	0,003031	6,527673
Príncipe Island								
Bumbo	East	0,42	Typical	5-14	2	Medium	1,602214	7,424071
Popa	East	0,10	Typical	1-4	1	Low	1,685980	7,429540
Cemitério	East	<0,05	Typical	5-14	4	Medium	1,567270	7,424220
Praia Grande	North	1,48	Typical	5-14	1	Low	1,670823	7,446521
Boi	North	0,34	Typical	5-14	1	Low	1,680463	7,459617
Ribeira Izé	North	0,51	Typical	1-4	4	High	1,685020	7,395001
Micotó	North	0,35	Typical	5-14	1	Low	1,681154	7,389707
Montanha	North	0,23	Typical	1-4	1	High	1,684137	7,393887
Sundy	North	0,42	Typical	5-14	5	High	1,679103	7,381196
Ponta Ramiro	North	0,11	Potential	<1	1	Medium	1,680326	7,378021
Margarida	North	0,07	Typical	1-4	2	Medium	1,680839	7,373861
Ponta Marmita	North	0,25	Typical	5-14	1	Low	1,682930	7,371763
Uba	North	<0,05	Typical	>15	2	Low	1,674352	7,458768
Macaco	North	0,60	Typical	1-4	4	High	1,681541	7,454099
Franguinha	North	0,14	Typical	1-4	0	Low	1,684029	7,450018
Banana	North	0,20	Typical	1-4	4	High	1,690216	7,441794
Campanha	North	0,26	Typical	<1	4	High	1,683507	7,426232
Bombom	North	1,32	Typical	1-4	4	High	1,690865	7,400796
Infante	South	1,40	Typical	5-14	1	Low	1,557488	7,413629
Praia Seca	South	0,56	Typical	5-14	4	High	1,545786	7,399314
Rio S. Tomé	South	0,46	Typical	1-4	2	Low	1,559279	7,354879

Key: NA number of activities*; IS* Impact Score*; HI* Human Impact*; LAT* Latitude*; LONG* Longitude

CHAPTER 5

GENERAL DISCUSSION

Diane Given Hayes

"In the end, we will conserve only what we love; we will love only what we understand and we will understand only what we are taught."

— Baba Dioum

GENERAL DISCUSSION

The general goals of this thesis were to contribute significantly for the assessment of the current conservation status of three species of marine turtles occurring in the islands of São Tomé and Príncipe. To achieve this, I addressed fundamental research questions about their reproductive biology, genetic structure and ecology, improving the current understanding of their population dynamics, connectivity with other populations, trophic niche in the region and reproductive behaviour. I used indirect methods that included the use of genetic markers such as microsatellites and mitochondrial DNA to inform on connectivity and dispersal in both green and olive ridley turtles; the use of stable isotopes to understand the trophic niches occupied by green turtles in São Tomé island, and modelling of reproductive behaviour data to understand the spatial and temporal distribution of the most important nesting aggregation of the critically endangered hawksbill turtle in the Eastern Atlantic. Here I highlight the key findings and discuss the broader conservation implications of this research for each of the three species.

Green turtle (*Chelonia mydas*)

Understanding a species distribution and the connectivity among geographically discrete populations is fundamental for its conservation and management. Genetic analysis has been vital for elucidating the historical processes that shaped the geographic distributions of several species and revealing their population structure (e.g. Bowen *et al.* 1994, 1997; Encalada *et al.* 1996; Dutton *et al.* 1999; Bowen & Karl 2007; Leroux *et al.* 2012; Naro-Maciel *et al.* 2014).

In **Chapter 2** of the thesis I used a combination of nuclear DNA markers (microsatellites) and mitochondrial DNA to evaluate the current levels of genetic diversity of *Chelonia mydas* and assess dispersal and recruitment of this species in São Tomé and Príncipe archipelago. We performed a mixed-stock analysis using sequences of both adult and juvenile turtles sampled during the study, as well as a compiled data set of several populations in the Atlantic. Both nuclear and $_{mt}$DNA data were congruent in showing that São Tomé and Príncipe's juvenile and adult green turtles exhibit high levels of genetic diversity and are both genetically differentiated from other foraging and nesting Atlantic populations. The mixed-stock analysis suggested that São Tomé and Príncipe's rookery is the primary source of juveniles to the local foraging areas, which suggests that green turtles in the archipelago show limited dispersal and should be considered a separate management unit for which conservation actions must be implemented, not only at the rookery level but also including the foraging aggregations.

The juvenile foraging aggregations were studied in further detail**,** as I explored the distribution of juvenile sea turtles of different life-stage groups in different habitats, as well as their trophic niche through in-water surveys and hand-capture of foraging individuals. I used the isotopic signatures of juveniles hand-captured at each foraging site to infer establishment duration at the foraging sites and trophic niches, and showed that juveniles establish local home ranges related to the available diet items, and use them for extended periods of at least several months. The variety of trophic roles fulfilled by juvenile green turtles must be taken into account; the contrasting consumption of items (seagrass vs. red algae) in relatively close foraging sites is an indication of plasticity in green turtle foraging behaviour in relation the available resources. Moreover, this study provided the first data set to which to compare demographic data from other locations in West Africa, where current knowledge on green turtle foraging behaviour is limited or non-existent and indicates that even oceanic islands that are geologically recent like São Tomé may provide important recruitment/development habitats for juvenile green turtles. For the green turtle, recruitment to the adult population occurs locally, thus the protection of its foraging habitats will provide additional conservation benefits.

Tagging data extracted from sea turtle databases maintained by the two on-site NGOs, and data obtained by daily and weekly surveys was combined and used to develop a stochastic model that can be used to estimate a critical parameter in life-history models and population estimates of sea turtles, the internesting period. I used the data obtained for *Chelonia mydas* to develop and test the model, and obtained an estimation of 12 days interval for this important parameter for the São Tomé and Príncipe population. The model described is an important step to understand patterns in the individual behaviour of females and how they affect the variation in internesting periods for a given population, and has applications for any marine turtle species.

Olive ridley turtle (*Lepidochelys olivacea*)

I genotyped a large number of females and hatchlings of Olive Ridley turtles (*Lepidochelys olivacea*) sampled during the study on São Tomé Island to study the reproductive behaviour and dispersal of this species in the region. The results, provided in **Chapter 3**, indicate male-biased dispersal, and a male-skewed operational sex-ratio. Knowing that low genetic diversity increases the risk of population extinction and may reduce adaptability to future environmental change, the current effective population size (Ne) and levels of nuclear genetic diversity were estimated to hypothesize about this population's ability to maintain adaptive potential in light of current high levels of exploitation, and potential future impacts of climate change. In São Tomé rookery, the results shows a male reproductive skew, and evidence of male biased

dispersal, as suggested by relatedness and mean assignment tests, which are adaptively advantageous traits. However, these life-history strategies appear to be insufficient to prevent the loss of genetic diversity as a result of a severe population bottleneck, and the estimated effective population size was much lower than the minimum needed to maintain equilibrium between loss of adaptative genetic variation due to genetic drift and its replacement through mutation. Because this species is considered a single large panmitic population sharing one common haplotype across the Atlantic Ocean, and genetic sampling of this species in the Atlantic is very limited, I could not assess population sub-structuring or cryptic population sub-divisions, and therefore with this study I could not hypothesize about future prospects of this species in São Tomé and Príncipe.

Hawksbill turtle (*Eretmochelys imbricata*)

I compiled all existing data available for *Eretmochelys imbricata* on both São Tomé and Príncipe in order to provide the first complete status assessment of this little known, yet critically and highly vulnerable population on the Eastern Atlantic. Data obtained from field observations were used to describe reproductive behaviour (nesting distribution, nest abundance and phenology) in **Chapter 4**. I showed that this species nests primarily in the southern beaches of São Tomé, while in Príncipe prefers the beaches in the north, and highlighted the importance of Rolas islet as the single most important nesting site for this species in the archipelago. This islet holds 71 % of all activity in São Tomé island, and 52.8 % of all activity in the archipelago, particularly in Joana and Marinho beaches, where I estimated a combined nesting activity of 225 ± 38.2 tracks during the 2016-2017 season. Based on range of estimates of the number of nests for each season and considering an average clutch frequency of 3 nests for this species, we estimate a minimum of 13 – 25 and a maximum of 25 – 34 individual females nesting in 2015-2016 and 2016-2017 seasons respectively in the whole archipelago. I found that a significant proportion of hawksbill nesting occurs either in well protected beaches, but even there some level of human impact can be scaled down. This is very important, as it is likely that this genetically isolated population may be composed of a very low number of females; for this reason, the loss or addition of one female nesting in one area in each year can significantly impact the population outcome.

FUTURE RESEARCH DIRECTIONS

Although this work focuses on the aggregations found in São Tomé and Príncipe archipelago, this study represents one of the most comprehensive genetic studies of green and olive ridley turtles in the Eastern Atlantic to date and provides the first data for several important life-history parameters for each species in the region. Nonetheless, to address some of the questions raised in this thesis more fully would require additional sampling, during more years and from other rookeries in the Atlantic. In particular this work should be extended to incorporate a significant proportion of the Eastern Atlantic rookeries to help inform a cohesive regional conservation strategy.

Traditionally mitochondrial DNA has been used to assess broad population structure among marine turtle rookeries (Bowen & Karl 2007; Jensen et al. 2013), but it is critical that the population structure of marine turtles is reassessed using genetic markers with a suitable variability, considering the demographic history and the geographic context of the studied populations (Bradshaw et al, 2018). This reassessment would be crucial to resolve the apparent isolation of the green and hawksbill sea turtles of São Tomé and Príncipe, and potential isolation of the olive ridley sea turtle as well, for which population dynamics in the Atlantic is still poorly understood. Genetic data should be complemented when possible with the attachment of electronic tracking devices, particularly to post-reproductive adults (including males), to define migratory routes between adult breeding and foraging areas, and to prioritise areas which can achieve the greatest conservation benefits for marine turtles. There has been very little satellite tracking of marine turtles in East Africa, and globally very little of males (but see van Dam *et al.* 2008).

Most marine turtle studies focus on adult females, which are more accessible; however, determining the number and movements of the breeding males should become a research priority as this has important implications for *Ne*, and for the adaptive potential and viability of marine turtle populations. In this thesis I used indirect methods for studying this population segment, in this case, of the olive ridley turtle; however future research efforts should attempt at collecting tissue samples and attaching platform terminal transmitters to males as well.

CONCLUSIONS

In summary, this thesis has significantly advanced our current knowledge on the ecology and connectivity of the São Tomé and Príncipe's marine turtle populations, shedding some light about the different species biology and ecology in East Africa. This study is in line with previous studies assessing marine turtle dispersal in the region, which show high genetic differentiation of the local rookeries of São Tomé and Principe due to very limited dispersal, highlighting the vulnerability of these populations to exploitation. This information is useful for informing a regional conservation strategy in order to adequately protect these species, particularly the green and hawksbill, as they should be considered two critically endangered subpopulations.

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